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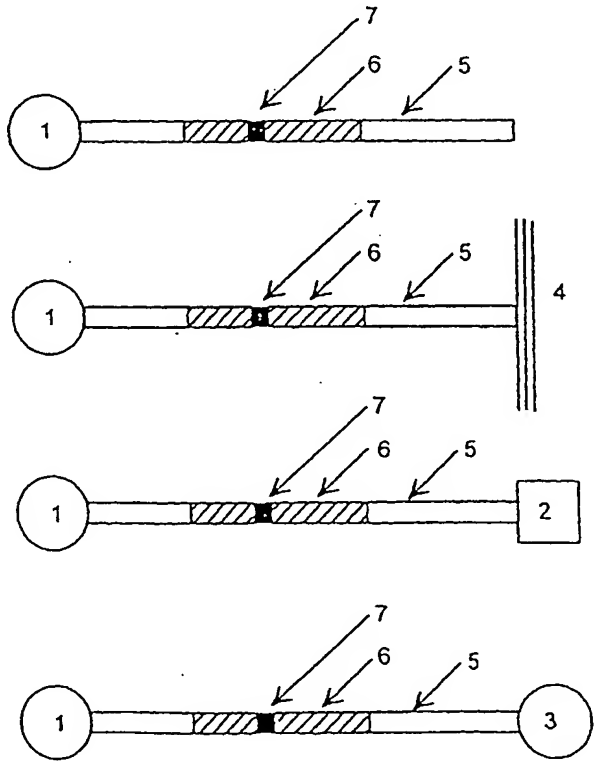
(54) Title: OPTICAL PROBES AND ASSAYS

(57) Abstract

This invention provides an optical probe useful as an optical probe or sensor of post translational type modifications, such as phosphorylation. The invention comprises a polypeptide moiety, which contains a recognition motif for a post translational type activity, and a protease site, which is coupled to a probe moiety. Modification of the polypeptide, by the post translational type activity, results in a modulation of the rate at which a protease cleaves the polypeptide which is sensed by a measurable change in at least one optical property of the optical probe upon cleavage. The present invention also includes a recombinant nucleic acid molecule that encodes an optical probe and a vector and host cell or library of cells that include the recombinant nucleic acid molecule. The optical probe can be used in methods to determine whether a sample, including a cell or a sample from an organism, contains a post-translational type modification activity. Such methods can also be used to determine whether a test chemical modulates the activity of a modifying activity, and thus can be used to identify therapeutic compositions. The identification of such therapeutic compositions can be automated using a system that includes an optical probe.



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OPTICAL PROBES AND ASSAYS

FIELD OF THE INVENTION

The present invention relates generally to the fields of chemistry and biology. More particularly, the present invention relates to optical probes for post translational type modification activities, such as phosphorylation, and methods for their use.

INTRODUCTION

Systems and methods for rapidly identifying chemicals with biological activity in samples, especially small liquid samples, is of particular relevance to the agrochemical and pharmaceutical fields. Various strategies are typically used to reduce processing times and associated costs of screening large numbers of chemical entities, including simplified assay design, automation, robotics and miniaturization of sample size. The advent of high throughput analysis and increasing use of miniaturized formats has led to the development of high density plate formats. For example, containing 384, 864 and 3456 wells as described in U.S. patent application No. 08/868,049 Entitled "*Low Background Multi-Well Plates with greater than 864 Wells for Fluorescence Measurements of Biological and Biochemical Samples*," filed July 3, 1997, now pending. Even higher density sample processing systems, for example using chips that contain miniaturized microfluidic devices are being developed (see, for example, R & D Magazine, November 1998, pages 38 to 43 entitled "*Lab-on-a Chip: Biotech's next California Gold Rush*").

Higher density plates enable faster analysis and handling of large sample or chemical libraries, such as in automated screening systems, but place considerable constraints on the assays that can be successfully employed within them. In particular, there is a need to develop assays that are compatible with miniaturized systems and which give accurate and reproducible assay results. Central to this need is a requirement for high sensitivity assays based on optical analysis, such as fluorescence or luminescence that do not require wash steps (e.g. "addition only assays").

One of the largest and most important classes of intracellular activities for which drugs may be particularly valuable are those involved in post-translational

modification activities. These activities are typically directed to the modification of proteins and nucleic acids within living cells to effect changes in the biological activity and function of these molecules. The major methods of protein or polypeptide, post-translational modification include protein phosphorylation, methylation, prenylation, glycosylation, ubiquitination sulfation and proteolysis (see generally *Cells. A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1998) review). Major methods of nucleic acid modification include methylation, ADP-ribsoylation and restriction digestion. A variety of environmental stimuli such as the presence of growth factors, hormones, changes in the cell cycle and toxins can transiently modulate the post-translational state of many intracellular components. The rapid development of specific, and effective inhibitors for a particular post-translational activity requires the development of suitable assays that can reliably and sensitively detect these activities in a high throughput screening system.

In spite of their great potential importance however, there are few existing methods of measuring such activities that are homogenous, non radioactive and sensitive enough to accurately and reproducible work in high throughput, or ultra high throughput screening systems. Such assays, by reducing the time required to identify and develop useful chemicals, can dramatically increase the value of a new drug by enabling its patentability and increasing it's period of exclusivity in the market.

Examples of such post-translational activities include, amongst others, protein methylation and prenylation. Protein prenylation involves the addition of isoprenoid moieties such as farnesyl and geranylgeranyl to proteins, and is a major mechanism of post-translational modification for many membrane-associated proteins. (Clark, 1992 *Protein isoprenylation and methylation at carboxyl-terminal cysteine residues*. Annu. Rev. Biochem. **61** 355-386). In most cases, the amino acid derivatized with the isoprenoid is a cysteine, or cysteines close to the carboxyl-terminus of the protein. Present methods of measuring protein prenylation and methylation typically involve labeling cells with radioactive precursors such as [^{3}H]-mevalonate or [^{3}H]-S-adenosylmethionine, isolation of the protein of interest and measurement of radioactive incorporation. There is thus a need for assays for these activities that are

sensitive, simple to use, non-radioactive and adaptable to high throughput screening methods.

Another important example of post-translational modification is protein glycosylation, which plays an extremely important role in the function of a significant number of proteins (Varki, 1993, *Biological roles of oligosaccharides*. Glycobiology 3 97-130). Protein glycosylation, unlike most other types of post-translational modification provides a wide diversity in the oligosaccharides added to a protein because of the potential for branching after the addition of the first sugar residue. Present methods of measuring glycosylation typically involve determining radioactive incorporation of a precursor oligosaccharide into a protein, isolating the protein and then measuring specific radioactive incorporation into a protein. There is thus a need for fluorescence or luminescence based assays for these activities that are adaptable to high throughput screening methods. It is one objective of the present invention to provide optical probes and methods of use that meet this need.

Protein kinases and phosphatases are generally recognized as one of the more important general mechanisms of regulating protein function. A recent review and analysis of diseases associated with genetic defects in protein kinases (www.nih.go.jp/mirror/pkr/pk_medicine.html) lists over 400 specific disease states associated with these activities alone. Protein kinases act on proteins via the addition of phosphate groups (phosphorylation) primarily on the amino acids, tyrosine, serine or threonine. Protein phosphatases in contrast, act to remove these phosphate groups thereby reversing the effects of phosphorylation. Changes in the phosphorylation state of proteins, can regulate the enzymatic activity, protein localization and protein-protein interactions of a particular protein within a cell. Such changes can subsequently modulate virtually every aspect of cellular metabolism, regulation, growth and differentiation. The overall balance of kinase and phosphatase activities in a cell is a primary determinant of the phosphorylation state of a protein at any one time.

However, current methods of measuring protein kinases, have many disadvantages, which prevents or hampers the ability to rapidly screen for drugs using miniaturized automated formats of many thousands of compounds.

For example, many current methods of measuring their activity rely on the incorporation and measurement of ^{32}P into the protein substrates of interest. In whole cells, this necessitates the use of high levels of radioactivity to efficiently label the cellular ATP pool and to ensure that the target protein is efficiently labeled with radioactivity. After incubation with test drugs, the cells must be lysed and the protein of interest purified to determine its relative degree of phosphorylation. This method requires large numbers of cells, long preincubation times, careful manipulation, and washing steps to avoid artifactual phosphorylation or dephosphorylation. Furthermore, this kinase assay approach requires purification of the target protein, and final radioactive incorporation into target proteins is usually very low giving the assay poor sensitivity. In high throughput screening operations, this approach requires large amounts of radioactivity, which can be an environmental and health hazard.

Alternative kinase assay methods, such as those based on phosphorylation-specific antibodies using ELISA-type approaches, involve the difficulty of producing antibodies that distinguish between phosphorylated and non-phosphorylated proteins.

Furthermore, most kinase measurements have the requirement for cell lysis, multiple incubations, and washing stages are time consuming, complex to automate, and potentially susceptible to artifacts.

There is thus a need for assays for enzymes, such as those involved in post-translational modification, that are sensitive, simple to use, applicable to virtually any activity and adaptable to high throughput screening methods. Preferably, such assays would not utilize radioactive materials so that the assays would be safe and not generate hazardous wastes. The present invention addresses these needs, and provides additional benefits as well.

SUMMARY OF THE INVENTION

This invention provides a fluorescent or bioluminescent substrate useful as an optical probe or sensor of post translational type modifications, such as phosphorylation. In one embodiment, the invention comprises a polypeptide moiety, which contains a recognition motif for a post translational type activity and a protease site, which is coupled to a probe moiety. Typically, the presence of a modification at

the recognition motif alters protease activity at the protease site resulting in a modulation of the cleavage rate of the protease. Cleavage is sensed by a measurable change in at least one optical property of the optical probe upon cleavage at the protease site, **FIG. 1**.

In one embodiment the probe is a fluorescent or luminescent moiety.

In another embodiment, the invention further comprises a fluorescent quencher coupled to the polypeptide that quenches emission from the first probe moiety. In this embodiment, the first probe moiety and the quencher moiety are coupled to the polypeptide such that the recognition motif and the protease site are located between them (**FIG. 1**). In this case, cleavage of the polypeptide by a protease results in an alteration in the fluorescence emission of the first probe moiety that may be used to determine post-translational activity.

In another embodiment, the optical probe may further comprise a second probe moiety coupled to the polypeptide that participates in energy transfer with the first probe moiety. In this embodiment, the first probe moiety and the second probe moiety are coupled to the polypeptide such that recognition motif and the protease site are located between them. In this case, cleavage of the polypeptide by a protease results in an alteration in energy transfer between the first probe moiety and the second probe moiety that may be used to determine post-translational activity.

The invention also provides methods for using the optical probes of the invention to determine whether a sample contains a post-translational type modification activity such as protein phosphorylation or dephosphorylation, methylation, prenylation or glycosylation. The method consisting of; i), contacting the optical probe with a sample, usually containing or suspected of containing a post translational type activity; ii), contacting the sample and optical probe with a protease, and iii), determining at least one optical property of said optical probe, or product thereof.

In another embodiment, the invention provides methods for using the optical probes of the invention to determine whether a test chemical modulates the activity of a post-translational type activity.

In another aspect, the invention provides a library of optical probes, each with a unique peptide sequence for use in selecting an optimal sequence specificity of a post-translational type activity.

Another aspect of the present invention includes a compound or therapeutic identified by at least one method of the present invention. These methods can include monitoring the efficacy and/or toxicology of said therapeutic in an *in vitro* or *in vivo* model. The compound can be provided in therapeutically acceptable carrier and can form a therapeutic composition.

A further aspect of the present invention includes various systems for spectroscopic measurements. In one embodiment, the system typically includes at least one reagent for an assay and a device, said device comprising a container and a platform. The container can include the optical sensor compounds of the present invention, and additional reagents necessary for the post-translational type activity. Addition of a sample to the container, followed by the addition of a protease after a given time results in a change in at least one fluorescent property of the optical probes of the present invention that can be used to determine the post-translational type activity of the sample.

In another embodiment the system can include a microfluidic spectroscopic system comprising at least one fluid containing structure with at least one electro-osmotic or electrophoretic system to control fluid movement within that structure.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and form part of the specification, merely illustrate embodiments of the present invention. Together with the

remainder of the specification, they are meant to serve to explain certain principles of the invention to those of skill in the art.

FIG. 1 Shows a schematic representation of some different embodiments of the present invention. In **FIG. 1**, a first probe moiety, **1**, is attached to a polypeptide, **5**, that comprises a post-translational modification recognition site for an activity, **6** (shown hatched in **FIG. 1**) and a protease site for a protease, **7**, (shown filled in **FIG. 1**). In one embodiment of the present invention, the optical probe may be attached to a solid surface, **4**, such as a bead. In another embodiment, the optical probe may further comprise a quencher, **2**, that is separated from the first probe moiety, **1**, by the polypeptide, **5**. In another embodiment, the optical probe may comprise a second probe moiety, **3**, that is again separated from the first probe moiety **1**, by the polypeptide, **5**.

FIG. 2 Shows fluorescence emission spectra of cleaved and non-cleaved of certain optical probes of the present invention. Dotted lines represent the spectra of the cleaved optical probe, and dashed lines represent the non-cleaved probe.

FIG. 3 Shows the dependency of 460/530 nm emission spectra ratios of certain optical probes (+/- kinase treatment) of the present invention upon incubation with increasing concentrations of a protease (chymotrypsin). Open symbols represent control samples, filled symbols represent phosphorylated samples, Squares represent the Src-1 substrate, triangles represent the Src-2 substrate and circles represent the Abl substrate.

FIG. 4 Shows the comparison of fluorescent changes mediated by certain optical probes of the present invention to ^{32}P-incorporation for the detection of inhibitors of tyrosine kinase activity. Triangles represent optical probe measurements, squares represent ^{32}P-incorporation measurements.

FIG. 5 Shows the detection of an inhibitor activity of protein tyrosine phosphatase activity by orthovanadate, using certain optical probes of the present invention.

FIG. 6 Shows a mock high throughput screening validation to verify that the present invention can be used to identify inhibitors of serine/threonine kinase activity.

FIG. 7 Shows the caspase-3 mediated cleavage of phosphorylated (filled symbols) and non-phosphorylated (open symbols) ERK kinase specific optical probes of the present invention.

FIG. 8 Shows the inhibition of ERK kinase activity by roscovitine using certain optical probes of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention recognizes that optical probes can be designed to act as optical sensors of post-translational activities through the creation of engineered molecules. In the present invention, post-translational modification of a polypeptide results in the modulation of the rate and efficiency of cleavage of the modified polypeptide compared to the non-modified peptide. The attachment of at least one probe moiety to the peptide couples the cleavage of the optical probe to a change in a fluorescence property of the substrate that may be used to determine the amount of post-translational activity in a sample, **FIG. 1.**

Abbreviations

t-Boc, tert-butyloxycarbonyl; Bzl, benzyl; CaMK, calmodulin dependent kinase; CKI, casein kinase 1; PDGF, platelet derived growth factor; Fmoc, fluorenylmethyloxycarbonyl; EGF, epidermal growth factor; ELISA, enzyme-linked immuno absorbant assay; FGF, fibroblast

growth factor; HF, hydrogen fluoride; HOBT, N-Hydroxybenzotriazole; PyBop, Benzotriazole-1-yl-oxy-tris-pyyrolidino-phosphonium hexafluorophosphate; TFA, trifluoroacteic acid.

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Definitions

Generally, the nomenclature used herein and many of the fluorescence, computer, detection, chemistry and laboratory procedures described below are those well known and commonly employed in the art. Standard techniques are usually used 10 for chemical synthesis, fluorescence, optics, molecular biology, computer software and integration. Generally, chemical reactions, cell assays and enzymatic reactions are performed according to the manufacturer's specifications where appropriate. The techniques and procedures are generally performed according to conventional methods in the art and various general references. (Lakowicz, J.R. *Topics in Fluorescence* 15 *Spectroscopy*, (3 volumes) New York: Plenum Press (1991), and Lakowicz, J. R. *Emerging applications of fluorescence spectroscopy to cellular imaging: lifetime imaging, metal-ligand probes, multi-photon excitation and light quenching*. Scanning Microsc Suppl Vol. 10 (1996) pages 213-24, for fluorescence techniques; Sambrook *et al. Molecular Cloning: A Laboratory Manual*, 2nd ed. (1989) Cold Spring Harbor 20 Laboratory Press, Cold Spring Harbor, N.Y., for molecular biology methods; *Cells: A Laboratory Manual*, 1st edition (1998) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., for cell biology methods; *Optics Guide 5* Melles Griot® Irvine CA, and *Optical Waveguide Theory*, Snyder & Love published by Chapman & Hall for general optical methods, which are incorporated herein by reference which are 25 provided throughout this document).

As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

The term "acceptor" refers to a quencher that operates via energy transfer. Acceptors may re-emit the transferred energy as fluorescence and are "acceptor 30 fluorescent moieties". Examples of acceptors include coumarins and related fluorophores, xanthenes such as fluoresceins, fluorescent proteins rhodols, and

rhodamines, resorufins, cyanines, difluoroboradiazaindacenes, and phthalocyanines. Other chemical classes of acceptors generally do not re-emit the transferred energy. Examples include indigos, benzoquinones, anthraquinones, azo compounds, nitro compounds, indoanilines, and di- and triphenylmethanes.

The term "bead" refers to a substantially spherical particle such as a sphere or microsphere. Beads may be used within a wide size range. Preferred beads are typically within the range of 0.01 to 100 μm in diameter. Beads may be composed of any material and may be substantially inert or comprise fluorescent, luminescence, electro-luminescent, chemo-luminescent, magnetic or paramagnetic probes. Such beads are commercially available from a variety of sources including Molecular Probes, Sigma or Polysciences.

The terms "cleavage site" or "protease site" refers to the bond cleaved by the protease (e.g. a scissile bond) and typically the surrounding three amino acids of either side of the bond. The letters "P_1", "P_2", "P_3" etc, refer to the amino acid positions, 1 amino acid, 2 amino acids and 3 amino acids N-terminal to the scissile bond. The letters "P'_1", "P'_2", "P'_3" refer to the amino acids positions 1 amino acid, 2 amino acids and 3 amino acids C-terminal to the scissile bond, as shown below;

Scissile bond

$$P_3\ P_2\ P_1 — P'_1\ P'_2\ P'_3$$

The term "engineered recognition motif" refers to a recognition motif that has been modified from the naturally existing sequence by at least one amino acid substitution.

The term "engineered protease site" refers to a protease site that has been modified from the naturally existing sequence by at least one amino acid substitution.

The term "fluorescent moiety" refers to a moiety that can absorb electromagnetic energy and is capable of at least partially remitting some fraction of that energy as electromagnetic radiation over some time period. Suitable fluorescent moieties include, but are not limited to, coumarins and related dyes, xanthene dyes such as fluoresceins, rhodols, and rhodamines, resorufins, cyanine dyes, bimanes,

acridines, isoindoles, dansyl dyes, aminophthalic hydrazides such as luminol, and isoluminol derivatives, aminophthalimides, aminonaphthalimides, aminobenzofurans, aminoquinolines, dicyanohydroquinones, semiconductor fluorescent nanocrystals, fluorescent proteins and fluorescent europium and terbium complexes and related compounds.

The term "fluorescent property" refers to any one of the following, the molar extinction coefficient at an appropriate excitation wavelength, the fluorescent quantum efficiency, the shape of the excitation or emission spectrum, the excitation wavelength maximum, or the emission magnitude at any wavelength during, or at one or more times after excitation of the fluorescent moiety, the ratio of excitation amplitudes at two different wavelengths, the ratio of emission amplitudes at two different wavelengths, the excited state lifetime, the fluorescent anisotropy or any other measurable property of a fluorescent moiety and the like. Preferably fluorescent property refers to fluorescence emission, or the fluorescence emission ratio at two or more wavelengths.

The term "homolog" refers to two sequences or parts thereof, that are greater than, or equal to 75% identical when optimally aligned using the ALIGN program. Homology or sequence identity refers to the following. Two amino acid sequences are homologous if there is a partial or complete identity between their sequences. For example, 85% homology means that 85% of the amino acids are identical when the two sequences are aligned for maximum matching. Gaps (in either of the two sequences being matched) are allowed in maximizing matching; gap lengths of 5 or less are preferred with 2 or less being more preferred. Alternatively and preferably, two protein sequences (or polypeptide sequences derived from them of at least 30 amino acids in length) are homologous, as this term is used herein, if they have an alignment score of more than 5 (in standard deviation units) using the program ALIGN with the mutation data matrix and a gap penalty of 6 or greater. See Dayhoff, M.O., *in Atlas of Protein Sequence and Structure*, 1972, volume 5, National Biomedical Research Foundation, pp. 101-110, and Supplement 2 to this volume, pp. 1-10.

The term "modulates" refers to either the enhancement or inhibition (e.g. attenuation of the rate or efficiency) partially or complete of an activity or process.

The term "modulator" refers to a chemical compound (naturally occurring or non-naturally occurring), such as a biological macromolecule (e.g., nucleic acid, protein, non-peptide, or organic molecule), or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian, including human) cells or tissues. Modulators are evaluated for potential activity as inhibitors or activators (directly or indirectly) of a biological process or processes (e.g., agonist, partial antagonist, partial agonist, inverse agonist, antagonist, antineoplastic agents, cytotoxic agents, inhibitors of neoplastic transformation or cell proliferation, cell proliferation-promoting agents, and the like) by inclusion in screening assays described herein. The activity of a modulator may be known, unknown or partially known.

The term "non-naturally occurring" refers to the fact that an object cannot be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring, while such a sequence that has been intentionally modified by man is non-naturally occurring.

The term "optical property" refers to a physical property of light, including the molar extinction coefficient at an appropriate excitation wavelength, the fluorescent or luminescent quantum efficiency, the shape of the excitation spectrum or emission spectrum, the excitation wavelength maximum or emission wavelength maximum, the ratio of excitation amplitudes at two different wavelengths, the ratio of emission amplitudes at two different wavelengths, the excited state lifetime, the fluorescent anisotropy or any other measurable optical property of a compound, or any product or emission derived from that compound, either spontaneously or in response to electrical or chemical stimulation or reaction.

The term "polypeptide" refers to a polymer in which the monomers are amino acids and are joined together through amide bonds, alternatively referred to as a

peptide. Additionally, unnatural amino acids, for example, beta-alanine, phenylglycine and homoarginine are also meant to be included. Commonly encountered amino acids, which are not gene-encoded, may also be used in the present invention. For a general review see Spatola, A.F., in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, B. Weinstein, eds., Marcel Dekker, New York, p. 267 (1983). All of the amino acids used in the present invention may be either the D- or L-isomer. The L-isomers are preferred. Chemically modified or substituted amino acids including phosphorylated, sulfated, methylated, or prenylated residues may also be used to create polypeptides for specific applications.

The term "post-translational type modification" refers to the enzymatic or non-enzymatic modification of an amino acid residue (preferably enzymatic). Such covalent modifications include phosphorylation, dephosphorylation glycosylation, methylation, sulfation, ubiquitination, prenylation and ADP-ribsoylation. Preferred post-translational type modifications include phosphorylation and dephosphorylation.

The term post-translational includes non-covalent type modifications including protein-protein interactions, and the binding of allosteric, or other modulators or second messengers such as calcium, or cAMP or inositol phosphates to the recognition motif.

The term "probe moiety" refers to a chemical moiety useful as a marker or indicator, or contrast agent for, absorption spectroscopy, luminescence spectroscopy, fluorescence spectroscopy, or magnetic detection.

The term "quencher" refers to a molecule or part of a compound that is capable of reducing the emission from a probe moiety. Such reduction includes reducing the light after the time when a photon is normally emitted from a fluorescent moiety. Quenching may occur by any of several mechanisms, including fluorescence resonance energy transfer, photoinduced electron transfer, paramagnetic enhancement of intersystem crossing, Dexter exchange coupling, and excitation coupling, such as the formation of dark complexes. Preferred quenchers include those that operate by fluorescence resonance energy transfer.

The term "recognition motif" refers to all or part of a polypeptide sequence recognized by a post-translational modification activity to enable a polypeptide to

become modified by that post-translational modification activity.. Typically, the affinity of a protein, e.g. enzyme, for the recognition motif is about 1 mM (apparent K_d), preferably a greater affinity of about 10 μM or less, more preferably, 1 μM or most preferably has an apparent K_d of about 0.1 μM. The term is not meant to be limited to optimal or preferred recognition motifs, but encompasses all sequences that can specifically confer substrate recognition to a peptide. Preferably the recognition motif is a phosphorylated recognition motif (e.g. includes a phosphate group), or other post-translationally modified residues. Typically the recognition motif will, at least partially, comprise a protease site. The protease site may be located at any location within recognition motif.

The term "test chemical" refers to a chemical to be tested by one or more screening method(s) of the invention as a putative modulator. A test chemical can be any chemical, such as an inorganic chemical, an organic chemical, a protein, a peptide, a carbohydrate, a lipid, or a combination thereof. Usually, various predetermined concentrations of test chemicals are used for screening, such as 0.01 micromolar, 1 micromolar and 10 micromolar. Test chemical controls can include the measurement of a signal in the absence of the test compound or comparison to a compound known to modulate the target.

Introduction

The present invention recognizes for the first time that optical probes can be designed to measure a range of post-translational type activities. The advantages of the present invention include compositions that can be used in methods, particularly methods for high throughput and miniaturized screening systems for drug discovery and profiling. Theoretical probes provide for assays, that typically exhibit a large dynamic range, increased sensitivity and allow ratiometric readouts for the detection of post-translational type activities.

As a non-limiting introduction to the breadth of the invention, the invention includes several general and useful aspects, including:

1) A polypeptide moiety, which contains a recognition motif for a post translational type activity and a protease site, which is coupled to a first fluorescent

moiety. Typically, the presence of a modification at the recognition motif alters protease activity at the protease site resulting in a modulation of the cleavage rate of the protease. Cleavage is sensed by a measurable change in at least one optical property of the optical probe upon cleavage at the protease site. In different embodiments, the invention may further comprise a second optical probe, such as fluorescent quencher or second fluorescent moiety or luminescent moiety. Typically the second optical probe is coupled to the polypeptide such that recognition motif and the protease site are located between them.

2) Methods for using the optical probes of (1) to determine whether a sample contains a post-translational type modification activity such as protein phosphorylation or dephosphorylation.

3) Methods for using the optical probes of (1) to determine whether a test chemical modulates the activity of a post-translational type activity.

4) Libraries of optical probes, each with a unique peptide sequence for use in selecting an optimal sequence specificity of a post-translational type activity.

5) A compound or therapeutic identified by at least one method of the present invention.

6) Systems for spectroscopic measurements using the optical probes (1) and methods above.

7) Microfluidic spectroscopic systems for using the optical probe (1) comprising at least one fluid containing structure with at least one electro-osmotic or electrophoretic system to control fluid movement within that structure.

These aspects of the invention and others described herein, can be achieved by using the methods and compositions of matter described herein. To gain a full appreciation of the scope of the invention, it will be further recognized that various aspects of the invention can be combined to make desirable embodiments of the invention. Such combinations result in particularly useful and robust embodiments of the invention.

Designing peptide sequences for use in the optical probes of the present invention.

Generally peptide sequences for measuring a post-translational type activity encompass a post-translational recognition motif that contains a residue that, when modified, modulates the rate of cleavage of the substrate by a protease as compared to the unmodified form. Typically, such peptides contain a single scissile bond (bond that is cleaved within the substrate) for a specific protease and exhibit reasonable solubility (e.g. 0.1 mg/ml or greater) in aqueous solution. The design and size of peptide sequences for specific optical probes, and the choice of a particular protease, is dependent upon the application for which the optical probe is to be used. For example, for resonance energy transfer type applications, the peptide separating the fluorescent or luminescent moieties will typically be in the range of 5 to 50 amino acids in length, preferably 10 to 25 amino acids in length, or more preferably 10 to 15 amino acids in length. For polarization based applications the peptide may be significantly larger, up to and including entire protein domains, for example 50 to 100 amino acids in length. Smaller peptides, in the range of 5 to 50 amino acids may also be used. Typically the protease site may be located at any position either completely or partially within the recognition motif. The recognition motif and protease site may be located at any position within the peptide with respect to the optical probe moiety. The section below describes the design of suitable peptide substrates for use in the present invention. Subsequent sections describe the selection and coupling of suitable fluorescent moieties for use in the invention. The following representative examples are offered by way of illustration, not by way of limitation.

A Design of peptides for measuring protein phosphorylation

In general protein kinases act on proteins via the addition of phosphate groups (phosphorylation) primarily on the amino acids, tyrosine, serine or threonine through a free hydroxyl group. The protein kinases that enzymatically catalyze these reactions may be classified into a number of distinct families based on shared

structural and functional properties. Typically, kinases within a family have a similar overall topology, have similar modes of regulation and have similar substrate specificity's (see, **Table 1**, and may be used with the invention, as well as those recognition motifs developed in the future). For example, members of the AGC (protein kinase A, G or C) families of kinases typically prefer phosphorylation recognition motifs with basic amino acids (R or K), those in the CMGC group typically prefer proline containing motifs, etc. In Table 1, blank cells in the "Substrate Preference" column indicate that the complete information for every member of a particular class was not available, or indicates that the family was too small to define a clear substrate preference, or indicates that no clearly defined substrate preference yet exists.

Within sub-families, particular members have specific preferences for amino acids at specific positions within the substrate. These preferences have been extensively characterized for a number of kinases as described herein. Additional methods for identifying the substrate specificities and binding recognition motifs of new kinases are known in the art and may be used with the present invention. Such methods enable the substrate specificity of virtually any kinase known now, or discovered in the future, to be rapidly identified, for example see U.S. patents No. 5,532,167 by Cantley *et al.*, issued July 2, 1996, and PCT application WO 98/54577 by Lai *et al.*, filed May 28, 1998.

TABLE 1

MAIN GROUPS	SUB-GROUPS	DESCRIPTION	SUBSTRATE PREFERENCE
AGC GROUP			
	Group 1	Cyclic nucleotide regulated protein kinase family	Arg/Lys Directed
	Group 2	Diacylglycerol-activated/phospholipid-dependent protein kinase family	Arg/Lys Directed
	Group 3	Related to protein kinase A and protein kinase C	Arg/Lys Directed
	Group 4	Kinases that phosphorylate G protein –coupled receptors	Negative charge Directed
	Group 5	Budding yeast AGC-related protein kinases	Not available
	Group 6	Kinases that phosphorylate	Arg/Lys Directed

		ribosomal protein S6 family	
	Group 7	Budding yeast DBF2/20 family	
	Group 8	Flowering plant PVPK1 protein kinase homologue family	
	Group 9	Other AGC related kinase families	Various
CAMK GROUP			
	CaMK Group 1	Kinases regulated by Ca^{2+}/CaM and close relatives	Arg/Lys Directed
	CaMK Group 2	CaMK group II	Arg/Lys Directed
	CaMK Other	Other CaMK related kinase families	Various
CMGC GROUP			
	CMGC Group 1	Cyclic-dependent kinases (CDKs) and close relatives family	Ser/Pro Directed
	CMGC Group 2	ERK (MAP) kinase family	Ser/Pro Directed
	CMGC Group 3	Glycogen synthase kinase 3 family	Ser/Pro Directed
	CMGC Group 4	Casein kinase II family	Negative charge directed
	CMGC Group 5	C1k family	
	CMGC Group 6	CMGC Group other	Various
PTK GROUP 1			
		Non-membrane spanning protein tyrosine kinases	
	PTK Group 1	Src family	IY directed
	PTK Group 2	Tec/Atk family	
	PTK Group 3	Csk family	IYM directed
	PTK Group 4	Fes (Fps) family	IYE directed
	PTK Group 5	Abl family	IYA directed
	PTK Group 6	Syk/ZAP70 family	YE directed
	PTK Group 7	Tyk2/Jak 1 family	
	PTK Group 8	Ack family	
	PTK Group 9	Focal adhesion kinase (Fak) family	
PTK GROUP 2			
		Membrane spanning protein tyrosine kinases	
	PTK Group	Epidermal growth factor receptor	EEEYF directed

	10	family (EGF)	
	PTK Group 11	Eph/Elk/Eck receptor	
	PTK Group 12	Axl family	
	PTK Group 13	Tie/Tek family	
	PTK Group 14	PDGF family	EEEYV directed
	PTK Group 15	FGF family	EXYXF directed
	PTK Group 16	Insulin receptor family	YMMM directed
	PTK Group 17	LTK/ALK family	
	PTK Group 18	Ros/Sevenless family	
	PTK Group 19	Trk/Ror family	
	PTK Group 20	DDR/TKT family	
	PTK Group 21	Hepatocyte growth factor receptor family	
	PTK Group 22	Nematode Kin 15/16 family	
	PTK Group 23	Other membrane spanning kinases	Various
OPK GROUP			
		Other protein Kinases (not falling in major groups)	
	OPK Group 1	Polo family	
	OPK Group 2	MEK/STE7 family	
	OPK Group 3	PAK/STE20 family	
	OPK Group 4	MEKK/STE11 family	
	OPK Group 5	NimA family	
	OPK Group 6	Wee1/mik1 family	
	OPK Group 7	Kinases involved in transcriptional control family	
	OPK Group 8	Raf family	

	OPK Group 9	Activin/TGFb receptor family	
	OPK Group 10	Flowering plant putative receptor kinases and close relatives	
	OPK Group 11	PSK/PTK "mixed lineage" leucine zipper domain family	
	OPK Group 12	Casein kinase 1 family	
	OPK Group 13	PKN prokaryotic protein kinase family	
	OPK Group 14	Other protein kinase families (each with no close relatives)	Various

Eukaryotic protein phosphatases are structurally and functionally diverse enzymes that are represented by three distinct gene families. Two of these, dephosphorylate phosphoserine and phosphothreonine residues, whereas the protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine amino acids. A subfamily of the PTPs, the dual specificity phosphatases, dephosphorylates all three phosphoamino acids. Within each family, the catalytic domains are highly conserved, with functional diversity endowed by regulatory domains and subunits.

The protein serine or threonine phosphatases type 1 and 2A account for as much as 95% of the phosphatase activity in cell extracts (Brautigan and Shriner, Methods. Enzymol. **159**: 339-346 (1988). These enzymes have broad substrate specificities and may be regulated *in vivo* through targeting of the enzymes to discrete sub-cellular localizations.

The total number of protein tyrosine phosphatases encoded in the mammalian genome has been estimated at between 500 and approximately 2000. These estimates are imprecise due to the large number of sequence database entries that are different splice forms or duplicates of the same PTP sequence.

i) Tyrosine phosphorylation or dephosphorylation

Optical probes for detecting tyrosine kinase activity according to the present invention are designed by incorporating the desired phosphorylation motif into a

peptide, and by ensuring that the only aromatic residue (Tyr, Trp or Phe) in the substrate is the tyrosine that is phosphorylated. It may also be preferable in certain cases to eliminate or reduce the number of negatively charged amino acids in the P'_1, P'_2 or P'_3 positions. If this is the case, then phosphorylation of the tyrosine residue by the tyrosine directed protein kinase activity modulates the rate of optical probe hydrolysis by chymotrypsin compared to the non-phosphorylated optical probe. The present inventors have recognized that elimination of negatively charged residues in the optical probe C-terminal to the scissile bond improves the efficiency of cleavage of non-phosphorylated optical probe, on occasion significantly increasing the utility of the optical probes for measuring kinase or phosphatase activities. This approach can be used to create specific optical probes for virtually all known tyrosine kinase activities by routine optimization of the reaction conditions as described herein. Specific illustrative examples for different tyrosine kinase classes are shown in **Table 2**, below for use with chymotrypsin.

TABLE 2

Kinase	Optimal recognition motif for the kinase	Optical probe specific motif
c-FGR	MEE**I**Y**G**IFF [2]	MEE**I**Y**G***ILS* SEQ. ID. NO: 1
Lyn	DEE**I**Y**E**ELE [2]	DEE**I**Y**E**SLE SEQ. ID. NO: 2
Src-1	GEEE**I**Y**G**EFEK [1]	GEEE**I**Y**G**EIEK SEQ. ID. NO: 3
C-Abl	AXV**I**Y**A**APF [1]	AEA**I**Y**A**AP*L* SEQ. ID. NO: 4
CSK	XEP**I**Y**M**FFF [2]	EP**I**Y**M**LSL SEQ. ID. NO: 5
Insulin receptor	XEEE**Y**MMMF [1]	EEE**Y**MMMM*M* SEQ. ID. NO: 6
PDGF receptor	EEEE**Y**VFIX [1]	EEEE**Y**V*V*IX SEQ. ID. NO: 7
EGF receptor	EEEE**Y**FELV [1]	EEEE**Y**V*L*LV SEQ. ID. NO: 8
FGF receptor	AEEE**Y**FFLF [1]	AEEE**Y**F*V*L*M* SEQ. ID. NO: 9

In **Table 2**, bold residues indicate those considered to be significant in kinase recognition, and italicized residues are those that can be substituted to enable effective modulation of the proteolytic sensitivity of the optical probe towards chymotrypsin upon phosphorylation. The tyrosine that is phosphorylated is underlined, and the

indicated references are (1) Songyang, *et al.*, Current Biology 4:973-983, 1994, and (2) Ruzzene, *et al.*, Eur. J. Biochem. 246: 433-439.

Optical probes for detecting protein tyrosine phosphatase activity according to the present invention are designed by incorporating the desired phosphorylation motif into a peptide, for example like those in (**Table 2**), or other such motifs developed now or in the future, and either enzymatically or chemically phosphorylating the appropriate amino acid. Dephosphorylation of the tyrosine residue in such optical probes by a tyrosine directed protein phosphatase activity modulates the rate of optical probe hydrolysis by chymotrypsin compared to the phosphorylated optical probe.

ii) Serine / threonine phosphorylation or dephosphorylation

To develop optical probes for measuring serine or threonine kinase activities, peptides are designed to incorporate a single aromatic amino acid (Tyr, Trp or Phe) that is typically located within about three amino acids of a serine or threonine residue, which is phosphorylated by an appropriate serine or threonine specific kinase. It is also preferable in certain cases (depending on the protease selected) to eliminate or reduce the number of negatively charged amino acids (e.g. Asp or Glu residues) in the P'_1, P'_2 or P'_3 positions to ensure that the effect of phosphorylation of the serine or threonine residue provides a large modulation in proteolytic sensitivity of the optical probe upon phosphorylation. Examples of such sequences are provided in **Table 3**, below, for use with chymotrypsin.

TABLE 3

Kinase	Optimal Motif	Optical probe with p-Ser in P'1	Optical probe with p-Ser in P'2
Protein kinase A	RRRR<u>S</u>IIFI [1]	RRRF<u>S</u>IIII SEQ. ID. NO: 10	RRFR<u>S</u>IIII SEQ. ID. NO: 11
Protein kinase C	RRRKF<u>S</u>FRRK[3]	RRRKF<u>S</u>LRRKA SEQ. ID. NO: 12	
CaMK I	LRRRL<u>S</u>*D*SNL [6]	LRRRF<u>S</u>*A*SNL SEQ. ID. NO: 13	
CaMK II	KRQQ<u>S</u>FDLF [2]	KRQF<u>S</u>IDLK SEQ. ID. NO: 14	KRFQ<u>S</u>IDLK SEQ. ID. NO: 15
Casein kinase I	F*D*TG<u>S</u>*I*IIFF [2]	GDQ*D*TY<u>S</u>*L*LDK	GDQ*D*YL<u>S</u>*L*DK

		SEQ. ID. NO: 16	SEQ. ID. NO: 17
Casein kinase II	EDEE**S**EDEE [2]	EDEF**S**EDEE SEQ. ID. NO: 18	EDFE**S**EDEE SEQ. ID. NO: 19
CycA/cdk2	HHHR***S***PRKR [1]	HHHF**S**PRKR SEQ. ID. NO: 20	HHFR**S**PRKR SEQ. ID. NO: 21
CycB/cdc2	HHHK**S**PRRR [1]	HHHF**S**PRRR SEQ. ID. NO: 22	HHFK**S**PRRR SEQ. ID. NO: 23
ERK	RVDEPD**S**PGEK[4]	RVDEPF**S**PGEK SEQ. ID. NO: 24	
Glycogen Synthase	PRPA**S**VPP [6]	PRPF**S**VPP SEQ. ID. NO: 25	
SLKI	RRFG***S***LRRF [1]	RRRF**S**LRRI SEQ. ID. NO: 26	RRFG**S**LRRI SEQ. ID. NO: 27
SRPK2	RRRH***S***RRRR [3]	RRRF**S**RRRR: SEQ. ID. NO: 28	RRRF**S**RRRR SEQ. ID. NO: 29

In **Table 3**, bold residues indicate those considered to be significant in kinase recognition, and italicized residues are those that can be substituted to enable effective modulation of the proteolytic sensitivity of the optical probe towards chymotrypsin upon phosphorylation. The serine that is phosphorylated is underlined, and the indicated references are (1) Songyang, *et al.*, Current Biology **4**: 973-983, (1994), (2) Songyang, *et al.*, Mol. Cell Biol. **16**: 6486-6493, (1996), (3) Wang, *et al.*, J. Cell Biol. **140**: 737-750, (1998), and (4)

Gonzalez *et al.*, J. Biol. Chem., **266**: 22159-22163, (1991). (5) Nishikawa et al., J. Biol. Chem. **272**: 952-960 (1997), (6) Kemp and Pearson Meth. Enzymology **200**: 121-155 (1991).

Optical probes for detecting protein serine or threonine phosphatase activity according to the present invention are designed by incorporating the desired phosphorylation motif into a peptide, for example like those in (**Table 3**), or other such motifs developed now or in the future, and either enzymatically or chemically phosphorylating the appropriate amino acid. Dephosphorylation of the serine or threonine residue in such an optical probe by a serine or threonine directed protein phosphatase activity modulates the rate of optical probe hydrolysis by chymotrypsin compared to the phosphorylated optical probe.

B Design of peptides for measuring protein prenylation.

Protein prenylation typically occurs through the addition of isoprenyl groups to cysteine residues located near the C-terminus of proteins. Typically the linkage of the isoprenyl moiety to cysteine occurs through the formation of a thioether with the cysteine sulfhydral. After the creation of the thioether intermediate, the modified protein may undergo proteolytic processing and cleavage to produce a product in which the cysteine is the C-terminal amino acid. A number of different isoprenylation activities have been identified that recognize distinct recognition motifs, as shown in **Table 4**.

TABLE 4

Description of Activity	Recognition motif	Examples of proteins modified
Farnesyl transferase [1]	.....CAAX	Lamins, p21[ras]
Type 1 geranylgeranyl transferase [1]	.....CAAL	Smgp21B, G-protein γ-subunit
Type 2 geranylgeranyl transferase [2]	.....CXC or XCC	Rab 3A

(1) Clarke, S. (1992) *Protein isoprenylation and methylation at carboxyl-terminal cysteine residues.* Ann. Rev. Biochem. 61: 355-386, (2) Kawata et al., *Post-translationally processed structure of the human platelet protein smg p21B: Evidence for geranylgeranylation and carboxyl methylation of the C-terminal cysteine.* Proc. Natl. Acad. Sci. **87:** 8960-8964 (1997). Optical probes for detecting protein prenylation activity according to the present invention are designed by incorporating the desired prenylation motif into a peptide, for example like those in (**Table 4**), or other such motifs developed now or in the future,
usually within three amino acids of the C-terminus. Additional amino acids may be incorporated N-terminal to the cysteine reside that is modified to enable subsequent coupling of a first probe moiety, provided that they do not introduce additional prenylation sites. Under these circumstances, prenylation of the optical probe results in an increase in the rate of cleavage of the substrate upon exposure to an isoprenylated protein-specific endoprotease. Such a protease activity results in the cleavage of the substrate between the modified amino acid and the adjacent amino

acid to liberate an intact tripeptide, and a new substrate with a C-terminal modified cysteine residue that results in a measurable change in at least one fluorescent property of the optical probe. Such a change can be used to measure protein prenylation activity as described in the section entitled *"Assays using optical probes"*.

5

C Design of peptides for measuring protein glycosylation

In general, oligosaccharides may be either N-linked or O-linked to a protein or 10 peptide. In the case of N-linked oligosaccharides, an N-acetylglucosamine residue is typically coupled to an asparagine residue. In the case of O-linked oligosaccharides, N-acetylgalactosamine is typically coupled to a serine or threonine residue. Optical probes for detecting N-linked protein glycosylation activity according to the present invention are designed by incorporating the desired glycosylation motif into a peptide, 15 for example like those in **(Table 5)**, or other such motifs developed now or in the future.

TABLE 5

Glycosylation Activity	Consensus Sequence motif
N-glycosylation [(1)]	NXT or NXS
O-Glycosylation [(2)]	X_1TPX_2P in preferred sequences X_1=uncharged, and X_2=small amino acids

(1) Gooley *et al.*, (1991) Biochem. Biophys. Res. Comm. **178:** (3) 1194-201; 20 and (2) Yoshida *et al.*, (1997) J. Biol. Chem. **272:** (27) 16884-8.

To provide the required modulation of proteolytic sensitivity of the substrate upon glycosylation the peptide should contain only one asparagine residue and no other basic amino acids such as lysine, arginine, histidine or glutamine residues. Under these conditions, the rate of cleavage of the substrate by trypsin is modulated 25 by N-linked glycosylation of the asparagine residue in the substrate, which can be coupled to an optical readout using the methods described herein for example in the section entitled "*Assays using optical probes*".

Choice of Protease

Generally proteases for use in the present invention typically have the following characteristics: They are commonly available at high purity, are substantially stable, and recognize a substrate recognition motif that comprises at least one position in which the presence, or absence, of a post-translationally modified residue modulates the activity of the protease towards that substrate.

Preferred substrates possess well defined protease sites, and exhibit a significant modulation e.g. at least 2 fold, or more preferably at least 5 fold modulation of activity towards a post-translationally modified residue compared to a non-modified residue.

A Choice of protease for measuring protein phosphorylation

Proteases that may be used to measure peptide phosphorylation or dephosphorylation include those that recognize a substrate recognition motif that comprises at least one position in which the presence or absence of a phosphorylated residue modulates the activity of the protease towards that substrate. For example like those in **(Table 6)**, or other such proteases developed now or in the future.

TABLE 6

Name	C number	Type	Peptide bond cleaved	Primary Specificity
Caspase 3		Cysteine	DXXD -P'_1	P_1=Asp, P'_1 = neutral preferred
Cathepsin G	C 3.4.21.20	Serine	P_1-P'_1	P_1 = aromatic preferred, W, Y, F
Chymotrypsin	C 3.4.21.1	Serine	P_1-P'_1	P_1 = aromatic preferred, W, Y, F
Elastase	C 3.4.21.36	Serine	P_1-P'_1	P_1 = uncharged, non aromatic, e.g. A, V, L, I, G, S, T P'_1 = non-specific
Endoproteinase Asp-N		Unknown	P_1-Asp	P'_1=Asp or P'_1=Cysteic acid P_1 = non-specific
Endoproteinase Glu-N	C 3.4.21.9	Serine	Glu- P'_1	P_1 = Glu or Asp P'_1 = non-specific
Streptomyces griseus GluSGP	C 3.4.21.82	Serine	Glu- P'_1	P_1 = Glu or Asp P'_1 = non-specific
Staphylococcus aureus V8	C 3.4.21.19	Serine	Glu- P'_1	P_1 = Glu or Asp P'_1 = non-specific

The flexibility in choice of phosphorylated amino acid (tyrosine, serine or threonine) combined with the flexibility in choice of the protease enables virtually any protein kinase or phosphatase activity to be measured using the present invention. It should be further noted that the above examples are illustrative of peptides that could be used to develop optical probes as described herein. Many other alternative substrates for a specific post-translational modification are possible by virtue of the inherent flexibility in the approach.

A contemplated version of the method is to use inducible controlling nucleotide sequences to produce a sudden increase in the expression of the protease within a cell, for the development of a cell based assay. An appropriate optical property would typically be monitored at one or more time intervals after the onset of increased expression of the protease.

B Choice of protease for measuring protein prenylation.

In the case of protein prenylation, proteases that exhibit modulated rates of cleavage of prenylated compared to non-prenylated substrates are preferred. For example, the yeast α factor maturation enzyme Ste24p Tam *et al.*, (1998) *Dual roles for Ste24p in yeast $_{\alpha}$factor maturation;NH2-terminal proteolysis and COOH-terminal CAAX processing.* J. Biol. Chem. **142**(3) 635-49; and the isoprenylated protein endoprotease; Ma *et al.*, (1992) *Substrate specificity of the isoprenylated protein endoprotease.* Biochemistry **31** (47) 11772-7, or other such proteases developed now or in the future.

C Choice of protease for measuring protein glycosylation

Preferred proteases for use in the present invention to measure N-linked glycosylation include enzymes that primarily recognize basic amino acids that can be modified by either enzymatic or non-enzymatic glycosylation reactions to create modified substrates with modulated rates of cleavage compared to non-modified substrates. For example, bovine trypsin, porcine trypsin and pineapple bromelian or other such proteases developed now or in the future. (see, Casey and

Lang (1976) *Tryptic hydrolysis at asparagine residues in globulin chains*. Biochim. Biophys. Acta 434: 184-8; Loh and Gainer, (1980) *Evidence that glycosylation of pro-opiocortin and ACTH influences their proteolysis by trypsin and blood proteases*. Mol. Cell. Endocrinol. (1) 35-44; Gil *et al.*, (1991), *Effect of non-enymatic glycosylation on reactivity in proteolysis.* Acta Cient Venez 42: (1) 16-23.)

Choice of Probe Moieties

The choice of the probe moiety is governed by a number of factors including, the type of measurements being made, the availability of specific instrumentation and the ease of coupling of the probe moiety to the peptide. Additionally, other factors that are specific to a particular application are also relevant and include, the effect of labeling on the solubility of the peptide, kinetics of the optical probe with respect to the post-translational activity or protease, and the required detection sensitivity of the assay. Fortunately numerous probe moieties are commercially available or can be readily made so that availability of probe moieties to meet a desired situation is not limiting.

For fluorescent probes, preferred fluorophores typically exhibit good quantum yields, lifetimes, and extinction coefficients, are resistant to collisional quenching and bleaching, and should preferably be easily conjugated to the ligand. Particularly desirable, are fluorophores that show absorbance and emission in the red and near infrared range, which are useful in whole animal studies, because of reduced scattering background fluorescence, and greater transmission through tissues. Examples of such moieties include cyanines, oxazines, thiazines, porphyrins, phthalocyanines, fluorescent infrared-emitting polynuclear aromatic hydrocarbons such as violanthrones, fluorescent proteins, near IR squaraine dyes. (For example as shown in Dyes and Pigments, 17 19-27 (1991), U.S. Patent No. 5,631,169 to Lakowicz *et al.*, issued May 20, 1997, and organo-metallic complexes such as the ruthenium and lanthanide complexes of U.S. patent nos. 4,745,076 and 4,670,572, the disclosures of which are incorporated herein by reference). The lanthanide complexes have the advantage of not being quenched by oxygen, and the long lifetimes may

allow easy suppression of the autofluorescence of biological samples. Specific materials include fluoroscein isothicyanate (especially fluorescein-5-isothiocyanate), dichlorotriazinylaminofluorescein, tetramethylrhodamine-5 (and –6)-isothiocyanate, 1,3-bis- (2-dialkylamino-5-thienyl)-substituted squarines, and the succinimidyl esters of: 5 (and 6) carboxyfluoroscein; 5 (and 6)-carboxytetramethylrhodamine; and 7-amino-4-methylcoumarin-3-acetic acid. Semiconductor fluorescent nanocrystals are available with a range of emission spectra, are highly fluorescent and are also preferred, (see Bruchez *et al.*, Science **281**: 2013-2016).

Preferred luminescent probes include chemi-luminescent, electro-luminescent and bioluminescent compounds. Preferred bioluminescent compounds include bioluminescent proteins such as firefly, bacterial or click beetle luciferases, aequorins and other photoproteins, for example as described in U.S. Patents 5,221,623, issued June 22, 1989 to Thompson *et al.*, and 5,683,888 issued November 4, 1997 to Campbell, 5,674,713 issued September 7 1997 to DeLuca *et al.*, 5,650,289 issued July 22, 1997 to Wood and U.S. patent 5,843,746 issued December 1, 1998 to Tatsumi *et al.* Preferred electro-luminescent probes include ruthenium complexes, as for example described in U.S. Patent 5,597,910 issued to Jan 28, 1997 to Gudibande. Preferred chemi-luminescent substrates include those based on 1,2-dioxetanes, as for example described in U.S. Patents 4,372,745 issued February 8, 1983 to Mandle *et al.*, 5,656,207 issued August 12, 1997 to Woodhead *et al.*, and 5,800,999 issued September 1, 1998 issued to Bronstein *et al.*

Preferred probes for use as NMR contrast agents include chelates of paramagnetic, ferromagnetic or diamagnetic metal ions complexed to lipophilic complexes as described in U.S. patents 5,628,982, issued May 13, 1997 to Lauffer *et al.* and U.S. patent 5,242,681, issued September 7, 1993 to Elgavish *et al.*, and fluorine-18-and 19 containing compounds J. Nucl. Med. **39** 1884-91 (1998).

In some applications it may be desirable to derivatize the compounds above to render them more hydrophobic and permeable through cell membranes. The derivatizing groups should undergo hydrolysis inside cells to regenerate the

compounds thus trapping them within cells. For this purpose, it is preferred that any phenolic hydroxyls or free amines in the dye structures are acylated with C_1-C_4 acyl groups (e.g. formyl, acetyl, n-butryl) or converted to various esters and carbonates, as described in Bundgaard, H., Design of Prodrugs, Elsevier Science Publishers (1985), Chapter 1, page 3 et seq., Further modification of the fluorescent moieties may also be accomplished, as required as described in U.S. patent No. 5,741,657 issued 4/21/98 to Tsien *et al.*

The probe may be attached to the polypeptide by a linker that provides a spacer between the probe and the peptide thereby preventing sterric interference of the probe on the interaction between the recognition motif and the post-translational-type activity. Preferred spacers are substantially stable under cellular conditions and easily coupled to the peptide and probe. Preferred examples include flexible aliphatic linkers such as γ-amino n-butyric acid (GABA), diaminopentane, and aminohexanoyl as well as rigid aromatic linkers. Such linkers are known in the art and described for example in the *Handbook of Fluorescent Probes and Research Chemicals*, by Richard Haugland, published by Molecular Probes.

Additionally non-covalent methods of attachment may also be used to label the peptide moiety. For example, the peptide may be designed to encompass a specific binding site for a fluorescent moiety as described in the pending U.S. Patent applications, identified by serial number 08/955,050, filed October 21, 1997, entitled *Methods of using synthetic molecules and target sequences*; 08/955,859, filed October 21, 1997, entitled *Synthetic molecules that specifically react with target sequences*, and 08/955,206, filed October 21, 1997, entitled *Target sequences for synthetic molecules*. Labeling may then be achieved by incubation of the peptide with the membrane permeate fluorescent binding partner, which has the advantages of enabling the expression of peptides within intact living cells, and the subsequent labeling of these peptides *in situ* to create optical probes within intact living cells.

Fluorescent proteins

For some cell based applications, preferred fluorescent moieties include endogenously fluorescent proteins, functional engineered fluorescent proteins, and homologs thereof. Because the entire fluorophore and peptide can be expressed

within intact living cells without the addition of other co-factors or fluorophores, such optical probes provide the ability to monitor post-translational activities within defined cell populations, tissues or an entire transgenic organism. For example by the use of inducible controlling nucleotide sequences to produce a sudden increase in the expression of the optical probe and suitable protease. Endogenously fluorescent proteins have been isolated and cloned from a number of marine species including the sea pansies *Renilla reniformis, R. kollikeri and R. mullerei* and from the sea pens *Ptilosarcus, Stylatula and Acanthoptilum*, as well as from the Pacific Northwest jellyfish, *Aequorea victoria*; Szent-Gyorgyi *et al.* (SPIE conference 1999), D.C. Prasher *et al.*, Gene, 111:229-233 (1992). These proteins are capable of forming a highly fluorescent, intrinsic chromophore through the cyclization and oxidation of internal amino acids within the protein that can be spectrally resolved from weakly fluorescent amino acids such as tryptophan and tyrosine.

Additionally fluorescent proteins have also been observed in other organisms, although in most cases these require the addition of some exogenous factor to enable fluorescence development. For example, the cloning and expression of yellow fluorescent protein from *Vibrio fischeri* strain Y-1 has been described by T.O. Baldwin *et al.*, Biochemistry (1990) 29:5509-15. This protein requires flavins as fluorescent co-factors. The cloning of Peridinin-chlorophyll *a* binding protein from the dinoflagellate *Symbiodinium* sp. was described by B.J. Morris *et al.*, Plant Molecular Biology, (1994) **24**:673:77. One useful aspect of this protein is that it fluoresces in red. The cloning of phycobiliproteins from marine cyanobacteria such as *Synechococcus*, e.g., phycoerythrin and phycocyanin, is described in S.M. Wilbanks *et al.*, J. Biol. Chem. (1993) **268**:1226-35. These proteins require phycobilins as fluorescent co-factors, whose insertion into the proteins involves auxiliary enzymes. The proteins fluoresce at yellow to red wavelengths.

A variety of mutants of the GFP from *Aequorea victoria* have been created that have distinct spectral properties, improved brightness and enhanced expression and folding in mammalian cells compared to the native GFP, **Table 7**, (*Green Fluorescent Proteins*, Chapter 2, pages 19 to 47, edited Sullivan and Kay, Academic Press, U.S. patent Nos: 5,625,048 to Tsien *et al.*, issued April 29, 1997; 5,777,079 to

Tsien *et al.*, issued July 7, 1998; and U.S. Patent No. 5,804,387 to Cormack *et al.*, issued September 8, 1998). In many cases these functional engineered fluorescent proteins have superior spectral properties to wild–type *Aequorea* GFP and are preferred for use in the optical probes of the invention.

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TABLE 7

Mutations	Common Name	Quantum Yield (Φ) & Molar Extinction (ε)	Excitation & Emission Max	Relative Fluorescence At 37 °C	Sensitivity To Low pH % max F at pH 6
S65T type					
S65T,S72A,N149K, M153T, I167T	Emerald	Φ = 0.68. ε = 57,500	487 509	100	91
F64L, S65T, V163A		Φ = 0.58 ε = 42,000	488 511	54	43
F64L,S65T (EGFP)	EGFP	Φ = 0.60 ε = 55,900	488 507	20	57
S65T		Φ = 0.64 ε = 52,000	489 511	12	56
Y66H type					
F64L,Y66H,Y145F, V163A	P4-3E	Φ = 0.27 ε = 22,000	384 448	100	N.D.
F64L,Y66H,Y145F		Φ = 0.26 ε = 26,300	383 447	82	57
Y66H,Y145F	P4-3	Φ = 0.3 ε = 22,300	382 446	51	64
Y66H	BFP	Φ = 0.24 ε = 21,000	384 448	15	59

Y66W type					
S65A,Y66W,S72A N146I,M153T, V163A	W1C	Φ = 0.39 ε = 21,200	435 495	100	82
F64L,S65T,Y66W, N146I,M153T, V163A	W1B	Φ = 0.4 ε = 32,500	434 452 476 (505)	80	71
Y66W,N146I, M153T, V163A	hW7	Φ = 0.42 ε = 23,900	434 452 476 (505)	61	88
Y66W			436 485	N.D.	N.D.
T203Y type					
S65G,S72A, K79R, T203Y	Topaz	Φ = 0.60 ε = 94,500	514 527	100	14
S65G,V68L,S72A T203Y	10C	Φ = 0.61 ε = 83,400	514 527	58	21
S65G,V68L,Q69K S72A, T203Y	h10C+	Φ = 0.71 ε = 62,000	516 529	50	54
S65G,S72A, T203H		Φ = 0.78 ε = 48,500	508 518	12	30
S65G,S72A T203F		Φ = 0.70 ε = 65,500	512 522	6	28
T203I type					
T203I, S72A, Y145F	Sapphire	Φ= 0.64 ε = 29,000	395 511	100	90
T203I T202F	H9	Φ = 0.6 ε = 20,000	395 511	13	80

Cell based Assays

Recombinant production of optical probes within living cells involves expressing nucleic acids having sequences that encode the fluorescent protein and substrate peptide as a fusion protein. In one embodiment described below, the optical probe comprises a first fluorescent protein, a peptide containing a post-translational modification recognition motif and a protease site, and a second fluorescent protein fused together as a single polypeptide chain. Nucleic acids encoding fluorescent proteins can be obtained by methods known in the art. For example, a nucleic acid encoding the protein can be isolated by polymerase chain reaction of cDNA from a suitable organism using primers based on the DNA sequence of the fluorescent protein. PCR methods are described in, for example, U.S. Pat. No. 4,683,195; Mullis

et al. (1987) Cold Spring Harbor Symp. Quant. Biol. 51:263; and Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989).

Suitable clones expressing the optical probes of the invention may then be identified, isolated and characterized by fluorescence activated cell sorting (FACS) typically enabling the analysis of a few thousand cells per second.

The construction of expression vectors and the expression of genes in transfected cells involve the use of molecular cloning techniques also well known in the art. Sambrook *et al.*, *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1989) and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., (Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (most recent Supplement). Nucleic acids used to transfect cells with sequences coding for expression of the polypeptide of interest generally will be in the form of an expression vector including expression control sequences operatively linked to a nucleotide sequence coding for expression of the polypeptide comprising the optical probe.

Methods of measurement

Methods that are preferred with the present invention include, fluorescence spectroscopy, luminescence spectroscopy, absorption spectroscopy and magnetic detection

Fluorescent methods that are preferred with the present invention include, continuous or time resolved fluorescence spectroscopy, fluorescence correlation spectroscopy, fluorescence polarization spectroscopy , and resonance energy based fluorescence spectroscopy. Methods of performing such assays on fluorescent materials are well known in the art and are described in, e.g., Lakowicz, J.R., *Topics in Fluorescence Spectroscopy*, volumes 1 to 3, New York: Plenum Press (1991); Herman, B., *Resonance energy transfer microscopy*, in: Fluorescence Microscopy of Living Cells in Culture, Part B, Methods in Cell Biology, vol. 30, ed. Taylor, D.L. & Wang, Y.-L., San Diego: Academic Press (1989), pp. 219-243; Turro, N.J., *Modern Molecular Photochemistry*, Menlo Park: Benjamin/Cummings Publishing Col, Inc. (1978), pp. 296-361. The selection and use of specific fluorophores or quenchers for

particular applications is known in the art, for example see, Berlman, I.B. *Energy transfer parameters of aromatic compounds*, Academic Press, New York and London (1973), that contains tables of spectral overlap integrals for the selection of resonance energy transfer partners. Additional information sources include the Molecular Probes Catalog, 1999; and Tsien *et al.*, 1990 *Handbook of Biological Confocal Microscopy* pp. 169-178.

ASSAYS USING OPTICAL PROBES

Methods for determining whether a sample has an activity typically involve contacting the sample with an optical probe, incubating the mixture under conditions to enable post translational modification of the substrate, and then adding a protease. Finally the degree of post-translational type activity in the sample is detected by determining at least one optical property of the optical probe or product thereof. In some cases, the optical probe and the protease may be added to a sample at the same time. Alternatively in the case where the sample contains cells, the method would typically involve stimulation of the cells, and then either lyzing the cells in the presence of the substrate, or in the case where the substrate is expressed within the cells, lyzing the cells in the presence of a protease to measure substrate modification. The method used to determine the degree of post-translational type activity is dependent on the assay format used.

In one aspect, the method may be based on the difference in fluorescence anisotropy of the optical probe before and after cleavage with a protease. In this case the optical probe typically comprises a polypeptide moiety, which contains a recognition motif for a post translational type activity and a protease site, which is coupled to a fluorescent moiety (**FIG. 1**). Modification of the polypeptide, by the post translational type activity, results in a modulation of the rate at which a protease cleaves the polypeptide which is sensed by a measurable change in fluorescence polarization of the optical probe upon cleavage.

Polarization measurements are based on the relative rotational movement of the fluorophore compared to the excited state life-time of that fluorophore. For globular molecules in dilute solution, the relationship between polarization (p) and the

degree of rotational movement can be readily derived (see Weber, *Polarization of the fluorescence of solutions*, in Fluorescence and Phosphorescence Analysis, Don Hercules (ed.), Interscience Publishers New York. Chapter 8, pages 217-240 (1966)). Rotational movement can be related to the rotational diffusion constant of the molecule, and hence to the molecular volume. In practice there is a close correlation between the molecular size and relative polarization of emitted light from a fluorophore. As a consequence, a significant change in fluorescence polarization can occur when the optical probes of the present invention are acted upon by a protease. Polarization based measurements are relatively easy to set up, and can be obtained over a wide concentration, temperature, and ionic strength range.

In one embodiment of this method, fluorescence anisotropy measurements may be enhanced by attaching one end of the peptide to a solid matrix, or a bead. In either case, cleavage of the optical probe results in a larger drop in fluorescence polarization because of the increased rotational flexibility of the optical probe once separated from the solid matrix or bead.

In another aspect, the present invention takes advantage of resonance energy transfer either between two fluorescent moieties (FRET), or a bioluminescent moiety and fluorescent moiety (bioluminescent resonance energy transfer, BRET), or a fluorescent moiety and a quencher (resonance energy transfer, RET) to provide a fluorescent readout.

In FRET applications, the optical probe typically comprises a first fluorescent moiety and a second fluorescent moiety coupled to the polypeptide such that the recognition motif and the protease site are located between them (**FIG. 1**). In this case, cleavage of the polypeptide by a protease results in an alteration in energy transfer between the first fluorescent moiety and the second fluorescent moiety that may be used to determine post-translational activity. In this case, the fluorescent moieties are typically chosen such that the excitation spectrum of one of the moieties (the acceptor fluorescent moiety) overlaps with the emission spectrum of the donor fluorescent moiety. The donor fluorescent moiety is excited by light of appropriate intensity within the donor fluorescent moiety's excitation spectrum and under

conditions in which direct excitation of the acceptor fluorophore is minimized. The donor fluorescent moiety then transfers the absorbed energy by non radiative means to the acceptor, which subsequently re-emits some of the absorbed energy as fluorescence emission, at a characteristic wavelength. FRET can be manifested as a reduction in the intensity of the fluorescent signal from the donor, reduction in the lifetime of its excited state, and an increase in emission of fluorescence from the acceptor fluorescent moiety. When the peptide substrate that connects the donor fluorescent moiety and acceptor fluorescent moiety is cleaved, the donor fluorescent moiety and the acceptor fluorescent moiety physically separate, and FRET is diminished or eliminated. Under these circumstances, fluorescence emission from the donor increases and fluorescence emission from the acceptor decreases.

The efficiency of FRET is dependent on the separation distance and the orientation of the donor fluorescent moiety and acceptor fluorescent moiety, the fluorescent quantum yield of the donor moiety and the energetic overlap with the acceptor moiety. Forster derived the relationship:

$$E = (F^0 - F)/F^0 = R_0^6/(R^6 + R_0^6)$$

where E is the efficiency of FRET, F and F^0 are the fluorescence intensities of the donor in the presence and absence of the acceptor, respectively, and R is the distance between the donor and the acceptor. R_0, the distance at which the energy transfer efficiency is 50%, of maximum is given (in Å) by

$$R_0 = 9.79 \times 10^3 (K^2 Q J n^{-4})^{1/6}$$

where K^2 is an orientation factor having an average value close to 0.67 for freely mobile donors and acceptors, Q is the quantum yield of the unquenched fluorescent donor, n is the refractive index of the intervening medium, and J is the overlap integral, which expresses in quantitative terms the degree of spectral overlap,

$$J = \int_0 F \, {}^4d \, / \int_0 F \, d$$

where is the molar absorptivity of the acceptor in M^{-1} cm^{-1} and F is the donor fluorescence at wavelength measured in cm. Forster, T. (1948) Ann.Physik 2: 55-75. The characteristic distance R_0 at which FRET is 50% efficient depends on the quantum yield of the donor, the extinction coefficient of the acceptor, the overlap between the donor's emission spectrum and the acceptor's excitation spectrum and the orientation factor between the two fluorophores.

Preferably, changes in the degree of FRET are determined as a function of the change in the ratio of the amount of fluorescence from the donor and acceptor moieties, a process referred to as "ratioing." By calculating the ratio, the assay is insensitive to fluctuations in substrate concentration, photobleaching and excitation intensity making the assay more robust. This is of particular importance in automated screening applications where the quality of the data produced is important for its subsequent analysis and interpretation.

A contemplated variation of the above assay is to either introduce, or express, the optical probe into living eukaryotic or prokaryotic cells to enable the measurement of intracellular post-translational activities.

In one aspect, the method would involve an optical probe comprising a first fluorescent protein, a peptide containing a post-translational modification recognition motif and a protease site, and a second fluorescent protein fused together as a single polypeptide chain. In this case the first fluorescent protein and the second fluorescent protein would be selected to enable FRET to occur as described above. A preferred pair of functional engineered fluorescent proteins for example being, Topaz (S65G, S72A, K79R, T203Y) and W1B (F64L, S65T, Y66W, N146I, M153T,V163A) (**Table 7**).

In another aspect the method would involve an optical probe comprising a peptide containing one or more binding sites for a fluorescent moiety, a post-translational modification recognition motif and a protease site. For example, the binding site could comprises a sequence that recognizes a fluorescent moiety as described in the pending U.S. Patent applications, identified by serial number

08/955,050, filed October 21, 1997, entitled *Methods of using synthetic molecules and target sequences*; 08/955,859, filed October 21, 1997, entitled *Synthetic molecules that specifically react with target sequences*, and 08/955,206, filed October 21, 1997, entitled *Target sequences for synthetic molecules*. In this case, expression of the peptide comprising the post-translational recognition motif, protease site and binding site could be accomplished using genetic means as described above. The addition of a membrane permeate fluorescent moiety capable of binding to the binding site would enable the creation, *in situ* of an optical probe.

In both cases, a contemplated version of the method is to use inducible controlling nucleotide sequences to produce a sudden increase in the expression of either the optical probe or the post-translational activity being assayed, e.g., by inducing expression of the construct. A suitable protease could be expressed within the cell, or induced, or introduced using a membrane translocating sequence U.S. patent No. 5,807,746, issued Sept 15 1998 to Lin *et al.* The efficiency of FRET is typically monitored at one or more time intervals after the onset of increased expression of the protease.

In another aspect the method would involve the introduction of an optical probe of the present invention into the cell through the use of a membrane translocating sequence, as described herein.

In BRET applications, the optical probe typically comprises a luminescent moiety and a fluorescent moiety coupled to the polypeptide such that the recognition motif and the protease site are located between them (**FIG. 1**). In this case, cleavage of the polypeptide by a protease results in an alteration in energy transfer between the luminescent moiety and the fluorescent moiety that may be used to determine post-translational type activities. In this case, the luminescent and fluorescent moieties are typically chosen such that the emission spectrum of the luminescent moiety overlaps with the excitation spectrum of the fluorescent moiety. Because the luminescent moiety provides light through a chemi-luminescent, electro-luminescent or bioluminescent reaction, there is no requirement for direct light excitation to create the excited state in the luminescent moiety. Instead appropriate substrates, or voltage must be provided to the luminescent moiety, to create an excited state within the

luminescent moiety. In the case of bioluminescent proteins, such substrates are, generically referred to as luciferins (for example see U.S. patent 5,650,289 issued July 22, 1997 to Wood). If BRET occurs, the energy from the excited state of the luminescent moiety is transferred to the fluorescent moiety by non radiative means, rather than being emitted as light from the luminescent moiety. Because the luminescent and fluorescent moieties emit light at characteristic wavelengths, the emission ratio of the two can provide a ratiometric readout as described for FRET based applications. BRET can be manifested as a reduction in the intensity of the fluorescent signal from the luminescent moiety, reduction in the lifetime of its excited state, and an increase in emission of fluorescence from the fluorescent moiety. When the peptide substrate that connects the luminescent moiety and fluorescent moiety is cleaved, the luminescent moiety and the fluorescent moiety physically separate, and BRET is diminished or eliminated. Under these circumstances light emission from the luminescent moiety increases and fluorescence emission from the fluorescent moiety decreases. The efficiency of BRET is dependent on the same separation and orientation factors as described above for FRET.

In RET applications, the optical probe typically comprises a first fluorescent moiety and a quencher moiety coupled to the polypeptide such that the recognition motif and the protease site are located between them (FIG. 1). In this case, cleavage of the polypeptide by a protease results in an alteration in energy transfer between the first fluorescent moiety and the quencher moiety that may be used to determine post-translational activity. In this case, the fluorescent moiety and the quencher moiety are typically chosen such that the absorption spectrum of one of the quencher (the acceptor moiety) overlaps with the emission spectrum of the donor fluorescent moiety. The donor fluorescent moiety is excited by light of appropriate intensity within the donor fluorescent moiety's excitation spectrum. The donor fluorescent moiety then transfers the absorbed energy by non radiative means to the quencher, which in this case does not re-emit any of the absorbed energy as light. RET can be manifested as a reduction in the intensity of the fluorescent signal from the donor or a reduction in the lifetime of its excited state. When the peptide substrate that connects the donor fluorescent moiety and quencher

moiety is cleaved, the donor fluorescent moiety and the quencher moiety physically separate, and RET is diminished or eliminated. Under these circumstances fluorescence emission from the fluorescent moiety increases.

The post-translational modification assays of the present invention can be used in drug screening assays to identify compounds that alter a post translational type activity. In one embodiment, the assay is performed on a sample *in vitro* (e.g. in a sample isolated from a cell, or cell lysate or purified enzyme) containing the activity. A sample containing a known amount of activity is mixed with an optical probe of the invention and with a test chemical. The amount of the activity in the sample is then determined after addition of a protease as described herein, for example, by determining at least one optical property of the probe. Then the optical property

of the sample in the presence of the test chemical is compared with the optical property of the sample in the absence of the test compound. A difference indicates that the test compound alters the activity.

In another embodiment, the ability of a test chemical to alter a post-translational type activity, in a cell based assay may be determined. In these assays, cells transfected with an expression vector encoding an optical probe of the invention, as described above, are exposed to different amounts of the test chemical, and the effect on FRET or fluorescence polarization in each cell can be determined after induction or introduction of a suitable protease. Typically, as with any method of the present invention, the difference in FRET or polarization of treated cells is compared to that of untreated controls.

Additionally libraries of optical probes can be created by producing peptides containing a diverse population of amino acid sequences. Such libraries are useful for the identification and characterization of novel post-translational type activities that have unknown or poorly defined substrate specificities.

As used herein, a "library" refers to a collection containing at least 5 different members, preferably at least 100 different members and more preferably at least 200 different members. The amino acid sequences for the peptide will typically be in the range or 10 to 20 amino acids in length and may be completely random or biased

towards a particular sequence based on a particular structural motif, for example based on a known substrate for a particular post-translational activity. In some instances the library will created genetically and the individual members expressed in bacterial or a mammalian cells. Suitable clones expressing the optical probes of the invention may then be identified, isolated and characterized by fluorescence activated cell sorting (FACS) typically enabling the analysis of a few thousand cells per second. Alternatively,, the peptides may be chemically synthesized and individual members attached to a solid matrix and arranged within a two dimensional array.

Typically, the library will contain variable peptides in which only a few, e.g., one to ten, amino acid positions are varied, but in which the probability of substitution is very high. Typically each member of the optical probe library will contain a single defined protease site, and a variable post-translational type recognition motif, such that randomized sequences comply with the design considerations for the particular post-translational type activity (described above). In one embodiment the array includes systematically substituted amino acids attached to a substrate, as described in U.S. Patent No. 5,770,456, issued June 23, 1998 to Holmes.

Screening of the library to identify optimal substrates may be achieved by incubating the array with a sample containing the post-translational activity, adding an appropriate protease, and then detecting at least one optical property from each member of the library. Those library members that are more efficiently modified by the post-translational type activity may then be identified by the degree to which the optical property of each library member is altered after exposure to the post-translational activity.

Alternatively libraries of known recognition motifs may be created in order to create an activity profile of post-translational activities in a sample. In this case, screening of the library is used to characterize the relative post-translational activities within by incubating the array with a sample containing the post-translational activities, adding an appropriate protease, and then detecting at least one optical property from each member of the library. Those library members that are more efficiently modified after exposure to the sample may then be identified by the degree

to which the optical property of each library member is altered after exposure to the sample to determine the post-translational activities present within the sample.

A System for Spectroscopic Measurements

The optical probes of the present invention can be used with various systems for spectroscopic measurement. In one embodiment, the system comprises: a reagent for an assay, and a device comprising at least one plate or container, preferably a multi-well platform, and a second platform to hold said plate or container for detecting a signal from a sample. The system can further comprise a detector, such as a detector appropriate for detecting a signal from a sample or a plate on in a container as such detectors are known in the art or are later developed. The system can comprise multiple plates or containers or multi-well platforms. In this context, a reagent for an assay includes any reagent useful to perform biochemical or biological *in vitro* or *in vivo* testing procedures, such as, for example, buffers, co-factors, proteins such as enzymes or proteases, carbohydrates, lipids, nucleic acids, active fragments thereof, organic solvents such as DMSO, chemicals, analytes, therapeutics, compositions, cells, antibodies, ligands, and the like. In this context, an active fragment is a portion of a reagent that has substantially the activity of the parent reagent. The choice of optical probe depends on the type of assay to be performed. For example, FRET based assays would typically comprise an optical probe with two fluorophores. Fluorescent polarization based assays would typically be completed with optical probes comprising one fluorescent moiety (**FIG. 1**).

The optical probes of the present invention are suited for use with systems and methods that utilize automated and integratable workstations for identifying modulators, and chemicals having useful activity. Such systems are described generally in the art (see, U.S. Patent Nos: 4,000,976 to Kramer *et al.* (issued January 4, 1977), 5,104,621 to Pfost *et al.* (issued April 14, 1992), 5,125,748 to Bjornson *et al.* (issued June 30, 1992), 5,139,744 to Kowalski (issued August 18, 1992), 5,206,568 Bjornson *et al.* (issued April 27, 1993), 5,350,564 to Mazza *et al.* (September 27, 1994), 5,589,351 to Harootunian (issued December 31, 1996), and

PCT Application Nos: WO 93/20612 to Baxter Deutschland GMBH (published October 14, 1993), WO 96/05488 to McNeil *et al.* (published February 22, 1996), WO 93/13423 to Agong *et al.* (published July 8, 1993) and PCT/US98/09526 to Stylli *et al.*, filed May 14, 1998.

Typically, such a system includes: A) a storage and retrieval module comprising storage locations for storing a plurality of chemicals in solution in addressable chemical wells, a chemical well retriever and having programmable selection and retrieval of the addressable chemical wells and having a storage capacity for at least 100,000 addressable wells, B) a sample distribution module comprising a liquid handler to aspirate or dispense solutions from selected addressable chemical wells, the chemical distribution module having programmable selection of, and aspiration from, the selected addressable chemical wells and programmable dispensation into selected addressable sample wells (including dispensation into arrays of addressable wells with different densities of addressable wells per centimeter squared) or at locations, preferably pre-selected, on a plate, C) a sample transporter to transport the selected addressable chemical wells to the sample distribution module and optionally having programmable control of transport of the selected addressable chemical wells or locations on a plate (including adaptive routing and parallel processing), D) a reaction module comprising either a reagent dispenser to dispense reagents into the selected addressable sample wells or locations on a plate or a fluorescent detector to detect chemical reactions in the selected addressable sample wells or locations on a plate, and a data processing and integration module.

The storage and retrieval module, the sample distribution module, and the reaction module are integrated and programmably controlled by the data processing and integration module. The storage and retrieval module, the sample distribution module, the sample transporter, the reaction module and the data processing and integration module are operably linked to facilitate rapid processing of the addressable sample wells or locations on a plate. Typically, devices of the invention can process at least 100,000 addressable wells or locations on a plate in 24 hours. This type of system is described in the PCT application WO/98/52047 by Stylli *et al.*, entitled "*Systems and method for rapidly identifying useful chemicals in liquid samples.*"

If desired, each separate module is integrated and programmably controlled to facilitate the rapid processing of liquid samples, as well as being operably linked to facilitate the rapid processing of liquid samples. In one embodiment the invention provides for a reaction module that is a fluorescence detector to monitor fluorescence. The fluorescence detector is integrated to other workstations with the data processing and integration module and operably linked with the sample transporter. Preferably, the fluorescence detector is of the type described herein and can be used for epi-fluorescence. Other fluorescence detectors that are compatible with the data processing and integration module and the sample transporter, if operable linkage to the sample transporter is desired, can be used as known in the art or developed in the future. For some embodiments of the invention, particularly for plates with 96, 192, 384 and 864 wells per plate, detectors are available for integration into the system. Such detectors are described in U.S. Patent 5,589,351 (Harootunian), U.S. Patent 5,355,215 (Schroeder), U.S. Patent Application (serial number pending), entitled "*Detector and Screening Device for Ion Channels*" filed 7/17/98, and PCT patent application WO 93/13423 (Akong). Alternatively, an entire plate may be "read" using an imager, such as a Molecular Dynamics Fluor-Imager 595 (Sunnyvale, CA). Multi-well platforms having greater than 864 wells, including 3,456 wells, can also be used in the present invention (see, for example, the PCT Application PCT/US98/11061, filed 6/2/98. These higher density well plates require miniaturized assay volumes that necessitate the use of highly sensitivity assays that do not require washing. The present invention provides such assays as described herein.

In another embodiment, the system comprises a microvolume liquid handling system that uses electrokinetic forces to control the movement of fluids through channels of the system, for example as described in U.S. patent No., 5,800,690 issued September 1, 1998 to Chow *et al.*, European patent application EP 0 810 438 A2 filed May 5 1997, by Pelc *et al.* and PCT application WO 98/00231 filed 24 June 1997 by Parce *et al.* These systems use "chip" based analysis systems to provide massively parallel miniaturized analysis. Such systems are preferred systems of spectroscopic measurements in some instances that require miniaturized analysis.

In another embodiment, the system may comprise a two dimensional array of optical probes dispersed on a substratum, for example as described in U.S. patents nos., 4,216,245 issued August 5, 1980 to Johnson, 5,721,435 issued February 24, 1998 to Troll, and 5,601,980 issued February 11, 1997 issued to Gordon *et al.* Such a system provides the ability to rapidly profile large numbers of optical probes and or large numbers of samples in a simple, miniaturized high throughput format.

A METHOD FOR IDENTIFYING A CHEMICAL, MODULATOR OR A THERAPEUTIC

The optical probes of the present invention can also be used for testing a therapeutic for useful therapeutic activity or toxicological activity. A therapeutic is identified by contacting a test chemical suspected of having a modulating activity of a biological process or target with a biological process or target on a plate or in a container, such as at least one well of a multi-well platform, that also comprises an optical probe. The test chemical can be part of a library of test chemicals that is screened for activity, such as biological activity. The library can have individual members that are tested individually or in combination, or the library can be a combination of individual members. Such libraries can have at least two members, preferably greater than about 100 members or greater than about 1,000 members, more preferably greater than about 10,000 members, and most preferably greater than about 100,000 or 1,000,000 members. After appropriate incubation of the sample with the optical probe, a protease is added and at least one optical property (such as FRET or polarization) of the sample is determined and compared to a non-treated control. If the sample having the test chemical exhibits increased or decreased FRET or polarization relative to that of the control or background levels, then a candidate modulator has been identified

The candidate modulator can be further characterized and monitored for structure, potency, toxicology, and pharmacology using well-known methods. The structure of a candidate modulator identified by the invention can be determined or confirmed by methods known in the art, such as mass spectroscopy. For putative modulators stored for extended periods of time, the structure, activity, and potency of the putative modulator can be confirmed.

Depending on the system used to identify a candidate modulator, the candidate modulator will have putative pharmacological activity. For example, if the candidate modulator is found to inhibit a protein tyrosine phosphatase involved, for example in T-cell proliferation *in vitro*, then the candidate modulator would have presumptive pharmacological properties as an immunosuppressant or anti-inflammatory (see, Suthanthiran *et al.*, Am. J. Kidney Disease, 28:159-172 (1996)). Such nexuses are known in the art for several disease states, and more are expected to be discovered over time. Based on such nexuses, appropriate confirmatory *in vitro* and *in vivo* models of pharmacological activity, as well as toxicology, can be selected. The optical probes, and methods of use described herein, enable rapid pharmacological profiling to assess selectivity and specificity, and toxicity. This data can subsequently be used to develop new candidates with improved characteristics.

BIOAVAILABILITY AND TOXICOLOGY OF CANDIDATE MODULATORS

Once identified, candidate modulators can be evaluated for bioavailability and toxicological effects using known methods (see, Lu, *Basic Toxicology, Fundamentals, Target Organs, and Risk Assessment*, Hemisphere Publishing Corp., Washington (1985); U.S. Patent Nos: 5,196,313 to Culbreth (issued March 23, 1993) and U.S. Patent No. 5,567,952 to Benet (issued October 22, 1996). For example, toxicology of a candidate modulator can be established by determining *in vitro* toxicity towards a cell line, such as a mammalian i.e. human, cell line. Candidate modulators can be treated with, for example, tissue extracts, such as preparations of liver, such as microsomal preparations, to determine increased or decreased toxicological properties of the chemical after being metabolized by a whole organism. The results of these types of studies are often predictive of toxicological properties of chemicals in animals, such as mammals, including humans.

The toxicological activity can be measured using reporter genes that are activated during toxicological activity or by cell lysis (see WO 98/13353, published 4/2/98). Preferred reporter genes produce a fluorescent or luminescent translational product (such as, for example, a Green Fluorescent Protein (see, for example, U.S.

Patent No. 5,625,048 to Tsien *et al.*, issued 4/29/98; U.S. Patent No. 5,777,079 to Tsien *et al.*, issued 7/7/98; WO 96/23810 to Tsien, published 8/8/96; WO 97/28261, published 8/7/97; PCT/US97/12410, filed 7/16/97; PCT/US97/14595, filed 8/15/97)) or a translational product that can produce a fluorescent or luminescent product (such as, for example, beta-lactamase (see, for example, U.S. Patent No. 5,741,657 to Tsien, issued 4/21/98, and WO 96/30540, published 10/3/96)), such as an enzymatic degradation product. Cell lysis can be detected in the present invention as a reduction in a fluorescence signal from at least one photon-producing agent within a cell in the presence of at least one photon reducing agent. Such toxicological determinations can be made using prokaryotic or eukaryotic cells, optionally using toxicological profiling, such as described in PCT/US94/00583, filed 1/21/94 (WO 94/17208), German Patent No 69406772.5-08, issued 11/25/97; EPC 0680517, issued 11/12/94; U.S. Patent No. 5,589,337, issued 12/31/96; EPO 651825, issued 1/14/98; and U.S. Patent No. 5,585,232, issued 12/17/96).

Alternatively, or in addition to these *in vitro* studies, the bioavailability and toxicological properties of a candidate modulator in an animal model, such as mice, rats, rabbits, or monkeys, can be determined using established methods (see, Lu, supra (1985); and Creasey, *Drug Disposition in Humans, The Basis of Clinical Pharmacology*, Oxford University Press, Oxford (1979), Osweiler, Toxicology, Williams and Wilkins, Baltimore, MD (1995), Yang, *Toxicology of Chemical Mixtures; Case Studies, Mechanisms, and Novel Approaches*, Academic Press, Inc., San Diego, CA (1994), Burrell *et al.*, *Toxicology of the Immune System; A Human Approach*, Van Nostrand Reinhld, Co. (1997), Niesink *et al.*, *Toxicology; Principles and Applications*, CRC Press, Boca Raton, FL (1996)). Depending on the toxicity, target organ, tissue, locus, and presumptive mechanism of the candidate modulator, the skilled artisan would not be burdened to determine appropriate doses, LD_{50} values, routes of administration, and regimes that would be appropriate to determine the toxicological properties of the candidate modulator. In addition to animal models, human clinical trials can be performed following established procedures, such as those set forth by the United States Food and Drug Administration (USFDA) or equivalents

of other governments. These toxicity studies provide the basis for determining the therapeutic utility of a candidate modulator *in vivo*.

EFFICACY OF CANDIDATE MODULATORS

Efficacy of a candidate modulator can be established using several art-recognized methods, such as *in vitro* methods, animal models, or human clinical trials (see, Creasey, supra (1979)). Recognized *in vitro* models exist for several diseases or conditions. For example, the ability of a chemical to extend the life-span of HIV-infected cells *in vitro* is recognized as an acceptable model to identify chemicals expected to be efficacious to treat HIV infection or AIDS (see, Daluge *et al.*, Antimicro. Agents Chemother., 41:1082-1093 (1995)). Furthermore, the ability of cyclosporin A (CsA) to prevent proliferation of T-cells *in vitro* has been established as an acceptable model to identify chemicals expected to be efficacious as immunosuppressants (see, Suthanthiran *et al.*, supra, (1996)). For nearly every class of therapeutic, disease, or condition, an acceptable *in vitro* or animal model is available. Such models exist, for example, for gastro-intestinal disorders, cancers, cardiology, neurobiology, and immunology. In addition, these *in vitro* methods can use tissue extracts, such as preparations of liver, such as microsomal preparations, to provide a reliable indication of the effects of metabolism on the candidate modulator. Similarly, acceptable animal models may be used to establish efficacy of chemicals to treat various diseases or conditions. For example, the rabbit knee is an accepted model for testing chemicals for efficacy in treating arthritis (see, Shaw and Lacy, J. Bone Joint Surg. (Br) 55:197-205 (1973)). Hydrocortisone, which is approved for use in humans to treat arthritis, is efficacious in this model which confirms the validity of this model (see, McDonough, Phys. Ther., 62:835-839 (1982)). When choosing an appropriate model to determine efficacy of a candidate modulator, the skilled artisan can be guided by the state of the art to choose an appropriate model, dose, and route of administration, regime, and endpoint and as such would not be unduly burdened.

In addition to animal models, human clinical trials can be used to determine the efficacy of a candidate modulator in humans. The USFDA, or equivalent

governmental agencies, have established procedures for such studies (see, www.fda.gov).

SELECTIVITY OF CANDIDATE MODULATORS

The *in vitro* and *in vivo* methods described above also establish the selectivity of a candidate modulator. It is recognized that chemicals can modulate a wide variety of biological processes or be selective. Panels of enzymes or panels of cells based on the present invention, or a combination of both, can be used to determine the specificity of the candidate modulator. Selectivity is evident, for example, in the field of chemotherapy, where the selectivity of a chemical to be toxic towards cancerous cells, but not towards non-cancerous cells, is obviously desirable. Selective modulators are preferable because they have fewer side effects in the clinical setting. The selectivity of a candidate modulator can be established *in vitro* by testing the toxicity and effect of a candidate modulator on a plurality of cell lines that exhibit a variety of cellular pathways and sensitivities. The data obtained from these *in vitro* toxicity studies can be extended into *in vivo* animal model studies, including human clinical trials, to determine toxicity, efficacy, and selectivity of the candidate modulator suing art-recognized methods.

For example arrays of kinase or phosphatase optical probes may be used to rapidly profile the selectivity of a test chemical with respect to its ability to inhibit related kinases or phosphatases. Such arrays may be located within a microtiter plate, or as a printed array, for example as disclosed in U.S. Patents Nos., 4,216,245 issued August 5, 1980 to Johnson, 5,721,435 issued February 24, 1998 to Troll, and 5,601,980 issued February 11, 1997 issued to Gordon *et al.* Such a system provides the ability to rapidly profile large numbers of kinases or phosphatases in the presence or absence of a test chemical in order to profile in a simple, miniaturized high throughput format the selectivity of a candidate modulator.

AN IDENTIFIED CHEMICAL, MODULATOR, OR THERAPEUTIC AND COMPOSITIONS

The invention includes compositions, such as novel chemicals, and therapeutics identified by at least one method of the present invention as having

activity by the operation of methods, systems or components described herein. Novel chemicals, as used herein, do not include chemicals already publicly known in the art as of the filing date of this application. Typically, a chemical would be identified as having activity from using the invention and then its structure revealed from a proprietary database of chemical structures or determined using analytical techniques such as mass spectroscopy.

One embodiment of the invention is a chemical with useful activity, comprising a chemical identified by the method described above. Such compositions include small organic molecules, nucleic acids, peptides and other molecules readily synthesized by techniques available in the art and developed in the future. For example, the following combinatorial compounds are suitable for screening: peptoids (PCT Publication No. WO 91/19735, 26 Dec. 1991), encoded peptides (PCT Publication No. WO 93/20242, 14 Oct. 1993), random bio-oligomers (PCT Publication WO 92/00091, 9 Jan. 1992), benzodiazepines (U.S. Pat. No. 5,288,514), diversomeres such as hydantoins, benzodiazepines and dipeptides (Hobbs DeWitt, S. *et al.*, Proc. Nat. Acad. Sci. USA 90: 6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, J. Amer. Chem. Soc. 114: 6568 (1992)), nonpeptidal peptidomimetics with a Beta-D-Glucose scaffolding (Hirschmann, R. *et al.*, J. Amer. Chem. Soc. 114: 9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen, C. *et al.*, J. Amer. Chem. Soc. 116:2661 (1994)), oligocarbamates (Cho, C.Y. *et al.*, Science 261: 1303 (1993)), and/or peptidyl phosphonates (Campbell, D.A. *et al.*, J. Org. Chem. **59**: 658 (1994)). See, generally, Gordon, E. M. *et al.*, J. Med Chem. **37**: 1385 (1994). The contents of all of the aforementioned publications are incorporated herein by reference.

The present invention also encompasses the identified compositions in a pharmaceutical composition comprising a pharmaceutically acceptable carrier prepared for storage and subsequent administration, which have a pharmaceutically effective amount of the products disclosed above in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985).

Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, acsorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used.

The compositions of the present invention may be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of nontoxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (e.g., liposomes), may be utilized.

The pharmaceutically effective amount of the composition required as a dose will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize. In practicing the methods of the invention, the products or compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These products can be utilized *in vivo*, ordinarily in a mammal, preferably in a human, or *in vitro*. In employing them *in vivo*, the products or compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. Such methods may also be applied to testing chemical activity *in vivo*.

As will be readily apparent to one skilled in the art, the useful *in vivo* dosage to be administered and the particular mode of administration will vary depending upon the age, weight and mammalian species treated, the particular compounds

employed, and the specific use for which these compounds are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, can be accomplished by one skilled in the art using routine pharmacological methods. Typically, human clinical applications of products are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved. Alternatively, acceptable *in vitro* studies can be used to establish useful doses and routes of administration of the compositions identified by the present methods using established pharmacological methods.

In non-human animal studies, applications of potential products are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear. The dosage for the products of the present invention can range broadly depending upon the desired affects and the therapeutic indication. Typically, dosages may be between about 10 mg/kg and 100 mg/kg body weight, and preferably between about 100 g/kg and 10 mg/kg body weight. Administration is preferably oral on a daily basis.
The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl *et al.*, *in The Pharmacological Basis of Therapeutics*, 1975). It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

Depending on the specific conditions being treated, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in *Remington's Pharmaceutical Sciences*, 18th Ed.,

Mack Publishing Co., Easton, PA (1990). Suitable routes may include oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art. Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed for the practice of the invention into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions of the present invention, in particular, those formulated as solutions, may be administered parenterally, such as by intravenous injection. The compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external micro-environment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. Determination of the effective amounts is well within

the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions. The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, for example, by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping, or lyophilizing processes. Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate. Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone,

carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses. Such formulations can be made using methods known in the art (see, for example, U.S. Patent Nos. 5,733,888 (injectable compositions); 5,726,181 (poorly water soluble compounds); 5,707,641 (therapeutically active proteins or peptides); 5,667,809 (lipophilic agents); 5,576,012 (solubilizing polymeric agents); 5,707,615 (anti-viral formulations); 5,683,676 (particulate medicaments); 5,654,286 (topical formulations); 5,688,529 (oral suspensions); 5,445,829 (extended release formulations); 5,653,987 (liquid formulations); 5,641,515 (controlled release formulations) and 5,601,845 (spheroid formulations).

EXAMPLES

The following examples are offered by way of illustration, not by way of limitation.

Example 1. Measurement of tyrosine kinase activity using optical probes.

Peptides were prepared by traditional solid-phase synthesis see, Merrifield, J. Amer. Chem. Soc., **85**:2149-2154 (1963); Fields, G. B., *et al.*, Principles and practice of solid-phase peptide synthesis, pages 77-183 in *Synthetic Peptides: A Users Guide,* Grant, G.R., ed., W. H. Freeman and Co. New York, (1992), in conjunction with the "tea-bag" methodology using Boc/benzyl based chemistry. See, Houghten *et al.*, Proc. Natl. Acad. Sci. USA **82**:513-5135 (1985). Peptides were assembled on methylbenzhydrylamine resin (MBHA resin) using traditional Boc/Benzyl based chemistry. A minor modification to the protocol (in the case of the abl-specific substrate (AEAIYAAPL, **SEQ. I.D. No. 4**) was the use of a base sensitive protecting

group (Fmoc) for the side chain of the C-terminal lysine residue. Bags, made of a polypropylene mesh material were filled with MBHA resin. The bags ("tea-bags") were placed in a Nalgene™ bottle with dichloromethane (DCM), enough to cover the bags, and shaken 5 min to allow the swelling of the resin. The DCM solution was then discarded and the actual synthesis was carried out. (All subsequent steps involved the addition of enough solvent to cover all the bags and vigorous shaking to ensure adequate solvent transfer).

The bags were washed 3 times, first with 5% diisopropylethylamine (DIEA) in DCM (neutralization step) for 2 minutes, and then twice with 100 % DCM (each for one minute) to remove excess base. After neutralization, the bags were sorted and placed into a Nalgene™ bottle containing a solution of the amino acid of interest in DCM, an equal amount of diisopropylcarbodiimide (DIC) in DCM was added to activate the coupling reaction. A 5-fold excess of amino acid and DIC was used for all of the couplings. The bottle was shaken for one hour to ensure completion of the reaction. The reaction mixture was discarded and the packets were washed in DMF twice for 1 minute to remove excess amino acids and by-products like diisopropylurea. Two final washes with DCM were performed to remove any excess DMF. The N-α-t-Boc was removed by acidolysis using a solution of 55% TFA in DCM for 30 minutes leaving the TFA salt of the α-amino group. The bags were washed successively with DCM (1 X 1 minute), isopropanol (2 x 1 minute) and DCM (1 X 1 minute). The synthesis was completed by repeating the same procedure while substituting for the corresponding amino acid at the coupling step.

After removal of the N-α-t-Boc from the γ-Amino-n-butyric acid (GABA), the bags were washed 3 times, 2 minutes each, with 5% DIEA in DCM, then with DCM (3 X 2 minutes). The bags were sorted, placed in a Nalgene™ bottle containing a solution of fluorescein isothiocyanate (FITC) in DCM/DMF (80/20) and shaken for 2 minutes (2-fold excess). Neat DIEA was then added to the FITC solution. The bottle was shaken for 3 hours to ensure completion of the reaction. The reaction mixture was discarded and the bags were washed in DCM (4 X 2 minutes) and DMF (1 X 2 minutes).

The Fmoc group on the side chain of the C-terminal lysine residue was removed using a solution of 20% piperidine in DMF for 25 minutes. The bags were washed successively with DMF (2 X 2 minutes), DCM (1 X 2 minutes) and DMF (1 X 2 minutes). Bags were then placed in a Nalgene™ bottle containing a solution of 7-hydroxycoumarin-3-carboxylic acid (1.5-fold excess) in DMF and shaken for 2 minutes. A solution of PyBop/HOBt in DMF was added to the bottle and the mixture was shaken for 2 minutes. Neat DIEA was then added and the mixture was shaken for 2 hours. The reaction mixture was discarded, the packets were washed with DMF (3 X 2 minutes) and DCM (3 X 2 minutes), and placed in a desiccator and dried under vacuum in preparation for cleavage.

All peptides were side chain deprotected and cleaved from the resin at 0°C with liquid HF in presence of anisole as a carbocation scavenger. The reaction was allowed to proceed for 60 minutes. Liquid HF was then removed using a strong flow of N_2 for 90 minutes followed by the use of aspirator vacuum for 60 minutes while maintaining the temperature at 0°C. The reaction vessels were removed from the apparatus and the residual anisole was removed with two ethylether washes. The peptides were extracted with two 30 ml 10% AcOH washes. For each peptide, the extraction solutions were pooled and lyophilized. The crude peptides were weighed and stored under nitrogen to await purification.

Automated Peptide Synthesis.

Alternatively, fluorescent peptide substrates were made using an automated peptide synthesizer (ABI 432A, Applied Biosystems, Foster City, CA) using Fmoc/ t-Boc chemistry. See Fields, G. B., *et al.*, Principles and practice of solid-phase peptide synthesis, pages 77-183 in *Synthetic Peptides: A Users Guide,* Grant, G.R., ed., W. H. Freeman and Co. New York, (1992). Briefly, after the automated peptide synthesis of the desired peptide (containing an unprotected N-terminal GABA and a Fmoc protected C-terminal lysine) was complete, the synthesis column was removed from the ABI432A synthesizer. The column containing the peptide attached to the resin was manually flushed with DMF to swell the resin. A two ml solution of 100 μM

FITC (5 fold excess) in 10% DIEA/DMF was then slowly injected through the synthesis column over a period of 2 hours using a syringe pump. The synthesis column was washed with (5 x 10 ml) DMF and THF (3 x 5 ml) and finally dried with a stream of dry nitrogen. The dried resin was suspended in 1 ml of trifluoroacetic (TFA) containing 50 µl of ethanedithiol and 50 µl of thioanisole, this mixture was stirred under nitrogen for 4 hours. The peptide was precipitated from the TFA solution by the addition of 20 ml of ether. The solid was further washed with ether (3 x 20 ml) to remove the thiol scavengers. The precipitated peptide (still mixed with the cleaved resin) was dried under vacuum. Finally, the C-terminal lysine of the peptide (in the case of the abl-specific peptide (AEAIYAAPL, **SEQ. ID. NO: 4**) was labeled with the N-hydroxysuccimidyl ester of 7-hydroxycoumarin-3-carboxylic acid (NHS-coumarin ester). This was accomplished by incubating a 5-fold excess of the NHS-coumarin ester with the peptide overnight at room temperature in a solution of DMF containing 10% DIEA. After removal of the solvent the peptide was purified as described below.

A fluorescein/rhodamine fluorescent substrate was produced using an identical procedure to that described above with the exception that an amine reactive rhodamine fluorophore (Lissamine rhodamine B sulfonyl chloride) was used to label the C-terminal lysine. Previous attempts to synthesize peptides with a C-terminal lysine labeled with rhodamine while the peptides were still attached to the resin were not successful. The method described above (labeling with rhodamine after cleavage from the resin) avoids the problematic tendency of rhodamine labels to bind irreversibly to the resins. Reaction of amine reactive rhodamine derivatives while the peptides are still attached to the resin apparently precludes them from reacting with the C-terminal lysine.

The crude peptides were purified by reversed-phase high-performance liquid chromatography on a C_{18} column using established methods. The mobile phase solvents were 0.1% TFA in water (Solvent A) and 0.1% TFA in Acetonitrile (Solvent B). The fractions containing the purified material were pooled and lyophilized and the purified peptides were characterized by analytical reverse phase-HPLC and by

mass spectral analysis. Peptide concentrations were determined by absorbance spectroscopy, using coumarin and fluorescein extinction coefficients of 35,000 and 75,000 M^{-1} cm^{-1}, respectively. Peptides were stable at 4 °C for at least one month and indefinitely at –20 °C.

Preparation of phosphorylated optical probes.

To prepare a sample of phosphorylated optical probe, the peptides were incubated with excess tyrosine kinase activity for a sufficient time to ensure complete phosphorylation of the peptide. Typically for v -Abl kinase reactions, the reaction buffer consisted of: 0.1x phosphate buffered saline (PBS), 5 mM $MgCl_2$, 200 μM ATP and not more than 10% of the total reaction volume of the tyrosine kinase enzyme. Reaction volumes were typically 20 μL, but were also performed at 10 μL and 100 μL. Recombinant v-Abl kinase was typically purchased from Calbiochem. Kinase reactions were quenched by the addition of 20 mM EDTA, pH 8. The degree of phosphorylation of the peptide was monitored over time by removing samples of the reaction mixture and analyzing them by reverse-phase high-performance liquid chromatography.

Alternatively, phosphorylated optical probes could be prepared directly during the peptide synthesis by simply using the O-benzyl protected phosphate derivative of the desired hydroxyl containing amino acid. For example, N α-Fmoc-O-benzyl-L-phosphotyrosine is commercially available and is compatible with standard Fmoc solid phase peptide synthesis. See White, P. *et al.* in "*Innovations & Perspectives in Solid Phase Synthesis and Combinatorial Libraries, 4th International Symposium*", R. Epton (Ed.), Mayflower Scientific Ltd. Birmingham, (1966), pp557. Thus, phosphorylated optical probes could be readily produced using protocols similar to those described above for automated peptide synthesis using Fmoc chemistry and purified as described below.

For example, using a Dionex HPLC apparatus and a C18 reverse-phase column by running a gradient elution profile consisting of either 5 to 80% acetonitrile / 0.1% trifluoroacetic acid (~pH 3) or 5 to 80 % acetonitrile / 0.1% triethylamine (~pH 7.5) over 25 minutes. Alternatively, the degree of phosphorylation was determined by

mass spectroscopy. Using both methods, the degree of peptide phosphorylation was typically greater than 95 % after incubation with the kinase. Negative control peptides were incubated under identical conditions to those for the phosphorylated peptides, but were incubated in the absence of ATP.

Fluorescence changes upon cleavage

To initially test cleavage of the optical probes, fluorescence emission measurements were made in a cuvette, using a steady-state fluorimeter (SPEX). In the case of fluorescein/coumarin labeled peptides, emission spectra between 420 and 600 nm were obtained by excitation at 405 nm, (where coumarin absorbs maximally and there is little direct excitation of fluorescein). Typically the concentration of the optical probes was 100 nM, and the total reaction volume was 700 μL.

FIG. 2 shows that cleavage of the non-phosphorylated optical probes by chymotrypsin results in a large increase in fluorescence emission at around 460 nm, and smaller decrease in emission at 530 nm that is caused by the loss of fluorescence resonance energy transfer (FRET) between the donor (coumarin) and acceptor (fluorescein). By comparison, the phosphorylated optical probe, is not degraded by chymotrypsin, and exhibits almost no change in emission characteristics at either wavelength upon incubation with the protease. The substantial 30-fold difference in emission ratios of phosphorylated (non-cleaved) substrate and non-phosphorylated (cleaved) substrate provides the basis for one aspect of the present invention. It should be further noted that since the emission spectra varies independently at two distinct wavelengths, it is possible to calculate an emission ratio, which has several significant advantages compared to single wavelength measurements. These include greater sensitivity and reproducibility in screening applications because the ratio is largely independent (within certain limits) of the absolute light intensity and optical probe concentration.

To confirm that the emission ratio is directly related to the degree of optical probe phosphorylation, mixtures of phosphorylated and non-phosphorylated peptides were mixed in defined amounts and diluted to 100 μL with 0.1X PBS and then added to a 96-well multiwell plate. Emission ratios (460/530) were acquired with a

Cytofluor plate reader (Perspective Biosystems) using a 395 nm excitation filter [full-width half-maximum (FWHM) of 25 nm] a 460 nm emission filter (FWHM=40 nm) and a 530 nm emission filter (FWHM=50 nm). Measurements were made before, and 1 minute after, addition of 0.04 nMol bovine alpha-chymotrypsin (Calbiochem, 230832, 1,018 USP units/mg), and the 460/530 emissions ratios calculated. The results, shown in **Table 8**, demonstrate a direct relationship between the degree of optical probe phosphorylation and the 460/530 emission ratio.

TABLE 8

% of Phosphorylated Peptide	Best Fit	460/530 Emission Ratio Actual Data
0	0.904	0.904
10	2.118	2.086
20	3.331	3.256
30	4.545	4.193
40	5.758	5.440
50	6.972	6.498
60	8.186	7.919
70	9.399	9.332
80	10.613	10.598
90	11.826	11.865
100	13.040	13.044

Optimization of protease concentration

To determine the relative proteolytic sensitivity of the phosphorylated and non-phosphorylated optical probe (AEAIYAAPL, **SEQ. ID. NO: 4**), samples of both were incubated with various concentrations of chymotrypsin, in 0.1 x PBS. Fluorescence measurements were made on a 96-well plate reader as described previously. In **FIG. 3**, the open symbols represent the control, non-phosphorylated optical probe. In this case, cleavage of the optical probe, as indicated by the 460/530 emission ratio, is already significant at 10 nM chymotrypsin and reaches a maximum value of around 12, in the presence of 100 nM protease under these conditions. By comparison, the phosphorylated optical probe (filled symbols) does not begin to exhibit a comparable change in emission ratio until exposed to a 1000-fold higher concentration of protease (10 μM). These results demonstrate that maximal

differences in emission ratio between phosphorylated and non-phosphorylated optical probe can occur, in this case, at protease concentrations between 0.1 to 1 μM chymotrypsin. Under these conditions, virtually all of the non-phosphorylated optical probe has been cleaved whereas virtually all of the phosphorylated optical probe is still intact. Optimal protease incubation conditions for other specific optical probes can be determined using similar procedures and protocols.

Example 2. Validation of optical probes for screening for protein tyrosine kinase inhibitors

To validate the invention in a high throughput screening format, optical probe-based assays were carried out in a 96-well plate reader. The results demonstrated highly reproducible and accurate results with the present invention. As shown in **Table 9,** the calculation of emission ratios significantly reduces the standard deviation and C.V. values compared to intensity measurements at either 460 or 530 nm. The reduction of errors is an important consideration in the design and analysis of screening systems, and particularly automated high throughput and ultra-high through screening systems.

TABLE 9

	Emission 460 nm	Emission 530 nm	Ratio
Mean	1290	1922	0.67
Standard Deviation	3.7	4.8	0.01
C.V.	2.9%	2.5%	1.5%

Analysis of the kinetics of phosphorylation of the optical probe revealed values for the apparent Km for the substrate of 40 μM, and an apparent Km for ATP of 8 μM. The turnover of the optical probe by v-Abl was 9.5 sec^{-1}, in agreement with published values for purified tyrosine kinases and optimal peptide substrates. For example, Seethala and Menzel., Anal Biochem. **253**: 210-218 (1997); Songyang *et al* ., Nature **373**: 536-539 (1995).

Comparison to other methods of screening.

To determine how the optical probe-based kinase assay compared to other screening methods, such as the direct measurement of ^{32}P-incorporation into a peptide, a direct comparison of the two methods was completed. Samples of fluorescent substrate (2 μM) were phosphorylated with v-Abl kinase as described above in either the presence, or absence, of γ-labeled ^{32}P- ATP (0.5 μM or 10 μCi per 20 μl reaction.) In the case of the ^{32}P-incorporation experiments, radioactive incorporation was determined by the binding of the optical probe to P81 filters as described previously (e.g., Seethala and Menzel, Anal. Biochem., **253**:210-218, 1997). Abl peptide binding to P81 filters proved to be able to reliably capture a constant fraction of phosphorylated peptide. Radioactive incorporation into the optical probe was monitored using a TopCount liquid scintillation counter (Packard). Incubation of the optical probe with chymotrypsin (100 nM) and measurement of fluorescence emission ratios were as described in ***Example 1***. Results from a typical experiment are shown in **Table 10**, and demonstrate that both methods of measuring tyrosine kinase activity give similar results and reliably indicate the level of substrate phosphorylation.

TABLE 10

Concentration of Enzyme (ng/assay)	% Phosphorylation as Determined by Fluorescence	% Phosphorylation as Determined by ^{32}P-Incorporation
0	0.0	0.0
0.5	3.1	4.6
1.5	9.0	12.3
4.0	36.8	42.1
10.0	69.2	65.6
22.0	96.0	95.5
44.0	100.0	100.0

Characterization of a protein tyrosine kinase inhibitor in a screening assay

To demonstrate that the present invention can effectively identify inhibitors of tyrosine kinase activity, a direct comparison was completed to compare the effect of an inhibitor of tyrosine phosphorylation on either fluorescence changes after incubation with chymotrypsin, or ^{32}P-incorporation (**FIG. 4**). The results

demonstrated almost identical dose dependencies and inhibition curves for the inhibitor using either method of measuring tyrosine kinase activity. These experiments therefore demonstrate that the present invention provides for a sensitive and convenient system of measuring phosphorylation, and that the results obtained with the assay system are directly comparable to those obtained with by measuring ^{32}P-incorporation.

Example 3. Measurement of other tyrosine kinase activities using optical probes

Measurement of Src kinase activity

To measure Src kinase activity, two optical probes Src-1 (GEEEIYGEIEK, **SEQ. ID. NO: 3**) and Src-2 (GEEEIYGVIEK, **SEQ. ID. NO: 29**) were developed. In the case of the Src-1 kinase substrate, and as shown in **Table 4**, a second aromatic amino acid was changed to isoleucine in the optical probe. In the second substrate, Src-2, the negatively charged amino acid (Glu =E) in the P'_2 position with respect to the protease site, was changed to valine (Val =V) to enable more efficient cleavage of the non-phosphorylated optical probe by chymotrypsin. Src kinase (Upstate Biotechnology) reaction conditions were the same as described in ***Example 1***, except 25 mM glycerol phosphate and 1 mM DTT were also added. Incubation of the optical probe with chymotrypsin (100 nM) and measurement of fluorescence emission ratios were as described in ***Example 1***. The apparent K_ms for the two substrates, (determined by fluorescence measurements after protease incubation) with respect to the Src kinase were 11 μM and 19 μM respectively. Other optical probes, designed as described herein, can be generated to create specific optical probes for a range of tyrosine kinase activities that can subsequently be optimized using the methods described herein.

Example 4. Measurement of protein tyrosine phosphatase activities using optical probes

To demonstrate that the present invention could also be used to determine protein tyrosine phosphatase activities, experiments were completed using phosphorylated optical probes incubated with protein tyrosine phosphatases. Optical

probes were first phosphorylated to completion as described above, and samples of phosphorylated optical probe, at a final concentration of 500 nM, were incubated with various concentrations of protein tyrosine phosphatase-B (PTP-B) agarose (Upstate Biotechnology) for 20 minutes at 30 C in 0.1X PBS. At the required time interval, PTP-B-agarose was removed by a brief microfuge spin prior to transfer to 96-well Cytofluor plates for fluorescence measurements, after addition of chymotrypsin (100 nM), as described in ***Example 1.***

Results from a typical experiment are shown in **Table 11.** In this experiment, the relative rates of dephosphorylation of the phosphorylated Src-2-specific substrate **(SEQ. ID. NO: 29)** and an abl-specific **(SEQ. I.D. No. 4)** substrate optical probes by the protein tyrosine phosphatase PTP-B were compared.

TABLE 11

Log concentration of protein tyrosine phosphatase [PTP-B]	Fluorescence emission ratio of Src-2 **(SEQ. ID. NO: 29)** peptide after protease treatment	Fluorescence emission ratio of abl **(SEQ. ID. NO: 4)** peptide after protease treatment
-7.7	17.7	n.d.
-8.7	17.6	17.7
-9.7	15.8	17.8
-10.7	5.40	16.6
-11.7	1.99	8.60
-12.7	1.75	5.70
-13.7	1.62	5.30
-14.7	1.60	5.30

In the case of the tyrosine phosphatase PTP-B, the Src-2 substrate is more readily de-phosphorylated than the abl substrate. Analysis of the enzyme kinetics, by virtue of a Michaelis-Menten plot, demonstrates that the apparent K_m for the Src-2optical probe is 1.3 μM and the k_{cat} is 79 sec^{-1}. The K_{cat}/K_m for this substrate is nearly 10^8 $M^{-1}sec^{-1}$, indicating extremely efficient recognition of the phosphotyrosine containing optical probe. These experiments therefore demonstrate that the present invention provides for a sensitive and convenient system of measuring protein

tyrosine phosphatase activity, and that the results obtained with the assay system are directly comparable to those obtained with other methods of measuring dephosphorylation. The relative broad substrate specificity of phosphotyrosine phosphatases (see, for example, Barford, *et al.* (1995) Nature Struct. Biol. **2:** 1043-1053), suggest that the approach will be useful for measuring a wide range of protein tyrosine kinase activities.

Example 5. Validation of optical probes for screening for protein tyrosine phosphatase inhibitors

To demonstrate that the present invention can be used to identify and characterize protein tyrosine phosphatase inhibitors, experiments were carried in the absence, or presence of various concentrations of orthovanadate, a well characterized competitive inhibitor of tyrosine specific phosphatase activities. Ortho-vanadate competitively inhibited tyrosine phosphatase activity with an apparent IC_{50} of 420 nM **(FIG. 5)** using the optical probes of the invention. This value is consistent with literature values of orthovanadate inhibition of PTP-B obtained by measuring ^{32}P-labeling. This result demonstrates that the present invention can be used for the development of sensitive and selective screening assays for the identification and characterization of protein phosphatase activities.

Example 6. Measurement of serine /threonine phosphorylation using optical probes

Measurement of protein kinase A activity

To demonstrate that optical probe could be developed to measure serine or threonine kinases, peptides was designed that could be effectively recognized and phosphorylated by protein kinase A. In this case, the substrate was designed with a single aromatic amino acid (F) that was located immediately N-terminal to the phosphorylation site for protein kinase A, underlined in SEQ. ID. NO: 12, below, (the P'_1 position with respect to the protease cleavage site of chymotrypsin). This results in a modulation of the rate of optical probe cleavage by chymotrypsin after phosphorylation.

The peptide (RRRKFSLRRKA, **SEQ. ID. NO: 12**) was labeled with fluorescein isothiocyanate at the N-terminus and 7-hydroxycoumarin-3-carboxamide at the C-terminus as described above. To determine the relative proteolytic activity of the phosphorylated and non-phosphorylated optical probes, samples of both were prepared. To do this, 10 microM of the substrate, in a total volume of 10 μL, was phosphorylated to completion by incubation with excess protein kinase A for one hour at 30 °C in a buffer consisting of 50 mM TRIS-Cl, pH 7.5, 10 mM $MgCl_2$, and 200 μM ATP. Mock kinase reactions with no ATP were used to create non-phosphorylated, control samples. In both cases, the samples were diluted 10 fold with buffer containing 50 mM HEPES, pH 7.5, 10 mM $CaCl_2$ and 0.01% Brij-35, and incubated with 0.8 nM chymotrypsin. Fluorescence emission ratios were monitored for one hour and are shown in **Table 12**.

TABLE 12

Time of Cleavage (minutes)	Non-phosphorylated Optical probe	Phosphorylated Optical probe	Fold Difference in Ratios
0	0.34 0.01	0.33 0.01	1.0
10	3.15 0.06	0.54 0.01	5.8
20	6.71 0.29	0.78 0.01	8.6
30	9.86 0.43	1.02 0.02	9.7
40	12.00 0.4	1.27 0.03	9.5
50	13.34 0.40	1.53 0.03	8.7
60	14.02 0.27	1.78 0.03	7.9

The maximum fold difference in 460/530 ratio was about 9.7 after 30 minutes of treatment with chymotrypsin. This ratio change therefore provides a robust and sensitive measure of protein phosphorylation that by virtue of its high signal to noise ratio is well suited for high throughput screening applications.

Example 7. Validation of optical probes for screening for serine/threonine kinase inhibitors

To demonstrate that the present invention could be used to identify and characterize serine/threonine kinase inhibitors, experiments were carried out with a number of previously characterized inhibitors of serine /threonine kinase activity. In the case of the ATP-competitive inhibitors staurosporin and H-89, inhibitor at a final concentration of 10 μM was preincubated with protein kinase A and the fluorescent substrate (**SEQ. ID. NO: 28**) in 50 mM TRIS-Cl, pH 7.5, 10 mM $MgCl_2$, and the reactions were initiated by the addition of ATP (10 μM). For the substrate competitive inhibitor PKI, inhibitor (2.8 μM) was pre-incubated with enzyme before the addition of optical probe and ATP (100 μM) in the buffer described above. After one hour incubation at 30 C, chymotrypsin to a final concentration of 0.8 nM was added and the 460/530 ratio was determined after one hour, as described above (***Example 6***). The results showed almost complete inhibition of protein kinase A activity at the concentrations of inhibitor tested (**Table 13A and 13B**).

TABLE 13A

Negative Control (No Active Kinase) 460/530 Emission Ratio	Positive Control (Active Kinase) 460/530 Emission Ratio	Kinase + the inhibitor Staurosporin (10 μM) 460/530 Emission Ratio	Kinase + the inhibitor H-89 (10 μM) 460/530 Emission Ratio
14.02 ± 0.27	1.78 ± 0.03	13.92 ± 0.07	13.29 ± 0.14

TABLE 13B

Negative Control (No Active Kinase) 460/530 Emission Ratio	Positive Control (Active Kinase) 460/530 Emission Ratio	Kinase + the inhibitor PKI (2.8 μM) 460/530 Emission Ratio
11.67 ± 0.48	2.11 ± 0.19	11.71 ± 0.15

Validation of the use of optical probes for high throughput screening

To demonstrate that the present invention could reproducibly detect inhibitors in a high throughput type-screening assay, a screen was performed in a 96-well plate.

The experiment was set up with randomly spiked wells containing a known protein kinase A inhibitor, staurosporin, under conditions where approximately 20 % of the substrate was converted to phosphorylated product. The 96-well plate was set up with appropriate no-ATP and no-inhibitor controls. Fifteen wells were chosen at random and received 5 µL of 360 nM staurosporin (in 3% DMSO). The final concentration of staurosporin in the five spiked wells was 60 nM, equal to the IC_{80} determined empirically. All other wells (including the no inhibitor wells) received 5 µL of 3% DMSO. All wells (except blanks) then received 10 µL of kinase reaction mix which included the optical probe (final concentration in the kinase reaction was 3.3 µM), buffer, and protein kinase A. After a 5 min pre-incubation, kinase reactions were started by the addition of 15 µL of 20 µM ATP, and incubated for 15 minutes at 30 C. Final kinase reaction concentrations were: 3.3 µM fluorescent substrate (**SEQ. ID. NO: 28**), 60 nM staurosporin, 0.004 units protein kinase A, 10 µM ATP, 10 mM $MgCl_2$. Kinase reactions were terminated by the addition of 30 µL of a buffer containing 50 mM HEPES, pH 7.4, 0.01% Brij-35, and 20 mM EDTA. Initial 460/530 ratios were obtained, and then the chymotrypsin reaction was started by the addition of 40 µL of a buffer containing 50 mM HEPES, pH 7.4, 0.01% Brij-35, and 2 nM chymotrypsin (50 ng/ml). Final chymotrypsin concentration in the reaction was 0.8 nM (20 ng/ml). 460/530 emission ratios were obtained after 30 minutes. In this assay format, all wells spiked with staurosporin were correctly assigned as positive hits for kinase inhibition (**FIG. 6**; filled in dots). Furthermore, in those wells, kinase activity was inhibited by about 80% when compared to the no ATP (negative) controls. The assay was highly reproducible, exhibiting a low coefficient of variance (**Table 14**).

TABLE 14

Samples	Coefficient of Variance
No ATP (n=7)	1.6%
Control Kinase (n=7)	2.6%
Kinase + Inhibitor (n=15)	1.8%
Kinase, no Inhibitor (n=65)	2.6%

Coefficient of Variance (in %) = 100 x (Standard Deviation/Mean)
N = number of wells

Example 8 Measurement of other serine threonine kinase activities

Measurement of casein kinase 1 activity

To measure casein kinase 1 activity, an optical probe was designed as described above. In this case, the kinase substrate was designed so that the point of phosphorylation was located at the P'_2 position (underlined in SEQ. I.D. NO: 17 below) with respect to the scissile bond cleaved by chymotrypsin, which enables the creation of a recognition motif suitable for casein kinase 1. A substrate peptide (GDQDYLSLDK, **SEQ. ID. NO: 17**) was synthesized and labeled with fluorescein isothiocyanate at the N-terminus and 7-hydroxycoumarin-3-carboxamide at the C-terminus as described in ***Example 1***.

Samples of phosphorylated and non-phosphorylated optical probes were prepared and tested as described in ***Example 1***. In this case, complete phosphorylation of the optical probe (1 μM) was obtained after room temperature incubation for 15 to 30 minutes in the presence of 500 Units of casein kinase 1 (New England BioLabs) in 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, and 200 μM ATP.

To determine the relative proteolytic sensitivity of phosphorylated and non-phosphorylated optical probes, samples of both were incubated with various concentrations of chymotrypsin and the cleavage monitored by measuring the 460/530 emission ratio as described in ***Example 1***. The results, shown in **Table 15**, demonstrate that the non-phosphorylated optical probe is significantly more susceptible to proteolytic cleavage at low concentrations of chymotrypsin than the phosphorylated optical probe.

TABLE 15			
Concentration of Chymotrypsin (μM)	Negative Control (No Active Kinase) 460/530 Emission Ratio	Positive Control (Active Kinase Only) 460/530 Emission Ratio	Fold difference in ratios
0.04	3.0	1.2	2.5
0.1	6.6	1.4	4.7
0.2	10.9	1.7	6.4
0.3	12.3	1.9	6.5
0.4	14.3	2.3	6.2
1.0	15.4	3.8	4.1
2.0	15.6	6.0	2.6

In this experiment, the maximum fold difference in 460/530 emission ratios of non-phosphorylated substrate versus phosphorylated substrate occurred at a chymotrypsin concentration of about 0.3 μM chymotrypsin. At this concentration the difference in emission ratios of phosphorylated and non-phosphorylated fluorescence samples was greater than 6 fold, demonstrating that the present invention provides for highly sensitive methods of measuring this class of serine/threonine kinase activities in a screening format.

Measurement of ERK kinase activity

To demonstrate that the present invention could also be used to detect the activity of a proline-directed serine / threonine kinase, an optical probe (VAPFSPGGRAK, **SEQ. ID. NO: 27**) was designed as a substrate for extracellular signal-regulated kinase (ERK) containing the serine phosphorylated, (shown underlined in SEQ. ID. NO: 27) in the P_1' position relative to the chymotrypsin cleavage site (F). This substrate (100 μM) was phosphorylated by incubation at 30 C for 3 hours in a 100 μL reaction containing 500 ng ERK (Biomol) and 500 μM ATP in a buffer consisting of 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, and 0.01% Brij-35. To test the proteolytic sensitivity of phosphorylated and non-phosphorylated samples, 2 μL of the mock (no kinase) or kinase reaction was diluted to 100 μL in a buffer containing 50 mM HEPES, pH 7.5, 10 mM $CaCl_2$, and 0.01% Brij-35, and incubated with chymotrypsin at a concentration of 4 nM. Cleavage

reactions were monitored on the Cytofluor for 1 hr. The results demonstrate that phosphorylated optical probe was less sensitive to chymotrypsin than the non-phosphorylated peptide (**Table 16**). The maximum fold difference in 460/530 ratio was about 15.2 and occurred after 30 minutes of cleavage. These data demonstrate that the present invention can be used to monitor activity of a proline-directed kinase.

TABLE 16

Time of cleavage (min)	Negative Control (No Active Kinase) 460/530 Emission Ratio	Positive Control (Active Kinase) 460/530 Emission Ratio	Fold difference in ratios
0	0.28	0.33	0.85
10	4.17	0.44	9.45
20	7.56	0.56	13.55
30	10.03	0.66	15.16
40	11.71	0.78	14.99
50	12.73	0.89	14.34
60	13.40	1.00	13.36

Measurement of protein kinase C activity

To measure protein kinase C activity using the optical probes of the present invention, a peptide (RRRKFSLRRKA, **SEQ. ID. NO: 12**) was designed in which phosphorylation by protein kinase C occurred at the P'_1 position (underlined in SEQ. ID. NO: 12) with respect to the scissile bond cleaved by chymotrypsin. This enabled an optimal protein kinase C recognition motif to placed within the optical probe sequence, and to create a site of phosphorylation that modulated the proteolytic sensitivity of the substrate towards chymotrypsin. Analysis of phosphorylated and non-phosphorylated samples of the optical probe revealed that phosphorylation by protein kinase C significantly modulated the proteolytic susceptibility of the substrate. These results demonstrate that the present invention can be used to develop optical probes that can measure protein kinase C activity.

Example 9. Measurement of serine/ threonine phosphatase activities

Protein Phosphatase I Activity

To determine if the optical probes could be used to detect serine/threonine phosphatase activities, samples of the casein kinase 1 specific phosphorylated optical

probes were prepared and treated with various serine/threonine protein phosphatases. To do this, the casein kinase 1 optical probe (GDQDYLSLDK, **SEQ. ID. NO: 17**) (100 μM) was phosphorylated to completion with CKI (2000 units) in a 100 μL reaction containing 200 μM ATP for 5 hours at 30 °C. A mock kinase reaction was performed which contained no ATP for preparation of control, non-phosphorylated optical probes. The resistance of the phosphorylated optical probe to chymotrypsin cleavage (data not shown) confirmed complete phosphorylation of the CKI-treated optical probe. Phosphorylated and non-phosphorylated samples of the optical probe (2 μM) were incubated in a 50 μL volume with or without 1 unit of the serine/threonine phosphatase protein phosphatase I (PPI) for 2 hours at 30 °C in a buffer consisting of 50 mM Tris-Cl, pH 7.0, 0.1 mM EDTA, 5 mM DTT, 0.01% Brij-35, and 1 mM $MnCl_2$. The reactions were diluted to 100 μL with 50 mM HEPES, pH 7.5, incubated with chymotrypsin (0.2 μM) for 1 hour at room temperature, and fluorescence values were measured on the Cytofluor as described above. Before chymotrypsin addition all reactions had similar 460/530 ratios of about 1.2.

TABLE 17

	Non-phosphorylated Control	Phosphorylated Control	Phosphorylated Control + Phosphatase (PP1)
460/530 Emission Ratio After Chymotrypsin	9.8	2.0	10.0

After chymotrypsin addition (**Table 17**) the 460/530 ratio of the non-phosphorylated optical probe was 9.8, whereas the 460/530 ratio of the phosphorylated optical probe was 2.0. However, when the phosphorylated optical probe was first treated with PP1 and then with chymotrypsin, the 460/530 ratio was 10.0, indicating that PP1 dephosphorylated nearly all of the optical probe.

Protein Phosphatase 2A Activity

The optical probes were also evaluated to determine if they could be used to measure protein phosphatase 2A (PP2A) activity. Phosphorylated and non-phosphorylated samples of the CKI optical probes (**SEQ. ID. NO: 23**) were prepared

and incubated with 0.03 units of PP2A as described above, except $MnCl_2$ was not included. After dilution and addition of chymotrypsin as described above, fluorescence values were measured using a 96 well plate reader (Cytofluor). After a 1.5 hour chymotrypsin incubation, the 460/530 emission ratio of the phosphorylated optical probe was 1.8 ± 0.2 **(Table 18).**

TABLE 18

	Non-phosphorylated Control	Phosphorylated Control	Phosphorylated Control + Phosphatase (PP2A)
460/530 Emission ratio After Chymotrypsin	11.0 ± 0.1	1.8 ± 0.2	8.8 ± 0.1

However, when the phosphorylated optical probe was first incubated with PP2A followed by chymotrypsin, the 460/530 ratio was 8.8 ± 0.1. This value is approximately 80% of that obtained when the non-phosphorylated optical probe was treated with chymotrypsin (11.0 ± 0.1). Thus, a majority of the phosphorylated optical probe was dephosphorylated under the conditions used. Taken together, these data indicate that the present invention can also be used as an assay for the activity of the serine/threonine phosphatase PP2A.

Example 10. Validation of optical probes for screening for serine or threonine protein phosphatase inhibitors

Identification of protein phosphatase 1 inhibitors

To determine if the optical probe protein phosphatase assay could detect inhibitors of PP1, the phosphatase assay was performed in the presence or absence of 1 μM microcystin-LR, a potent inhibitor of PP1. Phosphatase assays were set up as described above except the phosphatase was allowed to pre-incubate with microcystin-LR for 10 minutes before the addition of phosphorylated or non-phosphorylated optical probes **(SEQ. ID. NO: 23)**. PP1 reactions were incubated at 30 C for 1 hour and then diluted to 100 μL in 50 mM HEPES, pH 7.5 followed by the

addition of chymotrypsin to 0.2 μM. Fluorescence values were measured on the Cytofluor after a 2 hour incubation at room temperature as described above. After treatment of the phosphorylated optical probe with PP1 followed by chymotrypsin, the 460/530 ratio was 13.2 ± 0.2. This value was identical to that of the non-phosphorylated optical probe (13.2 ± 0.1) indicating that PP1 completely dephosphorylated the CKI-treated optical probe in this experiment (**Table 19**). However, in the presence of 1 μM microcystin-LR, PP1 activity was almost completely inhibited as demonstrated by the 460/530 ratio, which was 2.8 ± 0.1. Control samples, in which non-phosphorylated optical probe was treated with microcystin-LR gave a final 460/530 ratio of 13.3 ± 0.2, demonstrating that microcystin-LR did not inhibit chymotrypsin cleavage. Thus, the present invention could be used to detect inhibitors of PP1 activity.

TABLE 19

	Non-phosphorylated Optical probe + PP1	Phosphorylated Optical probe +PP1	Non-phosphorylated Optical probe + PP1 + microcystin-LR	Phosphorylated Optical probe + PP1 + microcystin-LR
After Chymotrypsin	13.2 ± 0.1	13.2 ± 0.2	13.3 ± 0.2	2.8 ± 0.1

Identification of protein phosphatase 2A inhibitors

To determine if the optical probe phosphatase assay could detect inhibitors of PP2A, the phosphatase assay was performed in the presence or absence of 100 nM microcystin-LR. PP2A assays were set up as described above, and were incubated at 30 C for 2 hours. Reactions were diluted to 100 μL with 50 mM HEPES, pH 7.5 followed by the addition of chymotrypsin to 0.2 μM. Fluorescence values were measured on the Cytofluor after a 1.5 hour incubation at room temperature. As described above, treatment of the phosphorylated optical probe (**SEQ. ID. NO: 23**) with PP2A followed by chymotrypsin gave a final 460/530 ratio of 8.8 ± 0.1. However, in the presence of 100 nM microcystin-LR, PP2A activity was completely inhibited as demonstrated by the 460/530 ratio of 1.9 ± 0.1 (**Table 20**). Control

samples in which non-phosphorylated optical probe was treated with microcystin-LR and chymotrypsin gave a final

460/530 ratio of 11.1 ± 0.2, demonstrating that microcystin-LR did not inhibit chymotrypsin cleavage. Thus, the optical probe based phosphatase assay can detect inhibitors of PP2A activity.

TABLE 20

	Non-phosphorylated optical probe + PP2A	Phosphorylated optical probe + PP2A	Non-phosphorylated optical probe + PP2A + microcystin-LR	Phosphorylated optical probe + PP2A + microcystin-LR
Before Chymotrypsin	1.4 ± 0.0	1.3 ± 0.0	1.4 ± 0.1	1.4 ± 0.1
After Chymotrypsin	11.1 ± 0.1	8.8 ± 0.1	11.1 ± 0.2	1.9 ± 0.1

Example 11. Use of other proteases to measure kinase activity

Measurement of ERK kinase activity

To determine how phosphorylation on serine in the optical probe (**SEQ. ID. NO: 24**) effected the rate of caspase-3 cleavage, samples of phosphorylated and control (non-phosphorylated) optical probes were treated with the protease caspase-3. Phosphorylated samples of the optical probe (70 pmol) were prepared by 8 hour to overnight incubation with ERK2 kinase (Biomol). Reactions were typically performed in 10 µl using 200-500 µM ATP and 50-200 ng ERK2 in a buffer consisting of 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT at 30°C. Mock kinase reactions were performed for preparation of control (non-phosphorylated) optical probe as above, except ATP was omitted. To monitor cleavage of the optical probes by caspase-3, 10 µL volumes of phosphorylated and control (non-phosphorylated) samples of the optical probe were placed in individual wells of a 96-well multiwell plate. Caspase-3 cleavage reactions were carried out in these samples after dilution to 100 µl in a buffer consisting of 100 mM HEPES, pH 7.5, 5 mM DTT, 0.5 mM EDTA, 20% glycerol, 0.01% Brij-35, and 50-100 ng

caspase-3 (Upstate Biotechnology), and incubated at room temperature. Emission readings were taken at 5 minute intervals during the course of the caspase-3 incubation using a Cytofluor plate reader as described in ***Example 1***.

As shown in **FIG.7**, the 460/530 emission ratio, which as described above, (***Example 1***) indicates increased cleavage of the optical probe, changes more rapidly for the control (non-phosphorylated) optical probe than it does for the phosphorylated substrate. These results demonstrate that phosphorylation of the optical probe by a serine /threonine directed protein kinase results in a modulation of the rate of cleavage of that substrate by, caspase-3. The maximal differences in fluorescence emission ratio occurred in this case after 30 minutes exposure to caspase-3, and resulted in over a three fold difference in emission ratio of phosphorylated and non-phosphorylated optical probes.

Measurement of serine or threonine kinase inhibitors

To confirm that the assay method could be used to detect inhibitors of ERK kinase activity, the effect of roscovitine (a known ERK kinase inhibitor) were examined using the present invention. To do this, ERK kinase (50 ng) was pre-incubated with the indicated amounts of roscovitine (Calbiochem), in the presence of 100 μM ATP. After 10 minutes optical probes (to a final concentration of 0.7 μM) were added and the incubations continued for an additional 2 h at 30°C. After incubation, reactions were diluted to 100 μl and fluorescence measurements made as described above in ***Example 6***.

The results show **FIG.8**, that the assay was able to detect the presence of the kinase inhibitor. The calculated IC_{50} for roscovitine using the optical probe based assay was 45 μM. These experiments therefore demonstrate that the present invention provides for a sensitive and convenient system of measuring Erk serine/threonine kinase inhibitor activity.

The present invention provides novel optical probes and methods for their use. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead

should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

SEQUENCE I.D. NO: LISTING.

SEQ. ID. NO: 1	MEEIYGILS
SEQ. ID. NO: 2	DEEIYESLE
SEQ. ID. NO: 3	GEEEIYGEIEK
SEQ. ID. NO: 4	AEAIYAAPL
SEQ. ID. NO: 5	EPIYMLSL
SEQ. ID. NO: 6	EEEYMMMM
SEQ. ID. NO: 7	EEEEYVVI
SEQ. ID. NO: 8	EEEEYVLLV
SEQ. ID. NO: 9	AEEEYFVLM
SEQ. ID. NO: 10	RRRFSIIII
SEQ. ID. NO: 11	RRFRSIIII
SEQ. ID. NO: 12	RRRKFSLRRKA
SEQ. ID. NO: 13	LRRRFSASNL
SEQ. ID. NO: 14	KRQFSIDLK
SEQ. ID. NO: 15	KRFQSIDLK
SEQ. ID. NO: 16	GDQDTYSLLDK
SEQ. ID. NO: 17	GDQDYLSLDK
SEQ. ID. NO: 18	EDEFSEDEE
SEQ. ID. NO: 19	EDFESEDEE
SEQ. ID. NO: 20	HHHFSPRKR
SEQ. ID. NO: 21	HHFRSPRKR
SEQ. ID. NO: 22	HHHFSPRRR
SEQ. ID. NO: 23	HHFKSPRRR
SEQ. ID. NO: 24	RVDEPFSPGEK
SEQ. ID. NO: 25	PRPFSVPP
SEQ. ID. NO: 26	RRRFSLRRI
SEQ. ID. NO: 27	RRFGSLRRI
SEQ. ID. NO: 28	RRRFSRRRR
SEQ. ID. NO: 29	RRFHSRRRR

We claim:

1. An engineered optical probe for measuring a post-translational type modification, activity comprising:

a polypeptide comprising a first fluorescent moiety,
wherein said polypeptide comprises a recognition motif for a post-translational type modification within said polypeptide, and
a protease site located within said polypeptide; and
wherein post translational type modification of said recognition motif modulates the rate of cleavage of said polypeptide by a protease with specificity for said protease site and said polypeptide is a non-naturally occurring polypeptide and said first fluorescent moiety permits detection of a polypeptide fragment.

2. The engineered optical probe of claim 1, wherein said polypeptide further comprises a second fluorescent moiety attached to said polypeptide, wherein said recognition motif and said protease site are located within said polypeptide between said first fluorescent moiety and said second fluorescent moiety.

3. The engineered optical probe of claim 1, wherein said polypeptide further comprises a luminescent moiety attached to said polypeptide, wherein said recognition motif and said protease site are located within said polypeptide between said first fluorescent moiety and said luminescent moiety.

4. The engineered optical probe of claim 1, wherein said polypeptide further comprises a quencher moiety attached to said polypeptide, wherein said recognition motif and said protease site are located within said polypeptide between said first fluorescent moiety and said quencher moiety.

5. The engineered fluorescent protein of claim 1, wherein said recognition motif is an engineered recognition motif.

6. The engineered fluorescent protein of claim 1, wherein said protease site is an engineered protease site.

7. The engineered optical probe of claim 1, wherein said polypeptide does not exceed about 15 amino acids in length.

8. The engineered optical probe of claim 1, wherein said polypeptide does not exceed about 50 amino acids in length.

9. The engineered optical probe of claim 5, wherein said polypeptide further comprises a quencher moiety, wherein said engineered recognition motif and said protease site are located within said polypeptide between said first fluorescent moiety and said quencher moiety.

10. The engineered optical probe of claim 5, wherein said polypeptide further comprises a second fluorescent moiety attached to said polypeptide, wherein said engineered recognition motif and said protease site are located within said polypeptide between said first fluorescent moiety and said second fluorescent moiety.

11. The engineered optical probe of claim 10, wherein said post-translational type modification is phosphorylation or dephosphorylation.

12. The engineered optical probe of claims 10, wherein said first fluorescent moiety comprises a first fluorescent protein or homolog thereof.

13. The engineered optical probe of claim 11, wherein said phosphorylation or dephosphorylation is phosphorylation a tyrosine kinase activity or dephosphorylation by a protein tyrosine phosphatase activity.

14. The engineered optical probe of claim 11, wherein said phosphorylation or said dephosphorylation is by a protein serine or threonine kinase activity or dephosphorylation by protein serine or threonine phosphatase activity.

15. The engineered optical probe of claim 11, wherein said phosphorylation or said dephosphorylation occurs at the P_1 position with respect to said protease.

16. The engineered optical probe of claim 11, wherein said phosphorylation or said dephosphorylation occurs at the P_2 position with respect to said protease.

17. The engineered optical probe of claim 12, wherein said fluorescent protein is an *Aequorea* green fluorescent protein.

18. A method for determining whether a sample contains a post-translational modification activity, comprising:

a) contacting a sample with an engineered optical probe, said engineered optical probe comprising:

a polypeptide comprising a first probe moiety,

wherein said polypeptide comprises a recognition motif for a post-translational type modification within said polypeptide, and

a protease site located within said polypeptide; and

wherein post translational type modification of said recognition motif modulates the rate of cleavage of said polypeptide by a protease with specificity for said protease site.

b) contacting said sample and said engineered optical probe with a protease with specificity for said protease site, and

c) detecting at least one optical property of said engineered optical probe or product thereof.

19. The method of claim 18, wherein said polypeptide does not exceed about 15 amino acids in length.

20. The method of claim 18, wherein said polypeptide does not exceed about 50 amino acids in length.

21. The method of claim 18, wherein said optical property comprises a fluorescent property.

22. The method of claim 21 wherein said fluorescence property is fluorescence anisotropy.

23. The method of claim 22, wherein said polypeptide is attached to a solid matrix.

24. The method of claim 18, further comprising a quencher attached to said polypeptide, wherein said recognition motif and said protease site are located between said first probe moiety and said quencher.

25. The method of claim 23, wherein said optical property is fluorescence emission.

26. The method of claim 25, wherein said polypeptide is attached to a bead.

27. The method of claim 18, further comprising a second probe moiety attached to said polypeptide, wherein said recognition motif and said protease site are located between said first probe moiety and said second probe moiety.

28. The method of claim 27, wherein said optical property is fluorescence resonance energy transfer.

29. The method of claim 25, wherein said optical property is bioluminescence resonance energy transfer.

30. The method of claim 25, wherein said protease is one of the following caspase 3, cathepsin G, chymotrypsin, elastase, endoproteinase Asp-N or endoproteinase Glu-N.

31. The method of claim 25, wherein said post-translational type activity is protein tyrosine kinase activity or protein tyrosine phosphatase activity.

32. The method of claim 25, wherein said post-translational type activity is protein serine or threonine kinase activity or protein serine or threonine phosphatase activity.

33. A method for determining whether a test chemical modulates the activity of a modifying activity, comprising the steps of:

contacting a sample containing an enzyme with a test compound and an engineered optical probe, said engineered optical probe comprising:

a first probe moiety attached to a polypeptide,

wherein said polypeptide comprises a recognition motif for a post-translational type modification activity, and

a protease site located within said polypeptide; and

wherein post translational type modification of said recognition motif within said polypeptide by said post-translational type modification activity modulates the rate of a protease with specificity for said protease site to cleave said polypeptide,

b) contacting said sample containing an enzyme with a test compound and said engineered optical probe with a protease, and

c) determining at least one optical property of said engineered optical probe.

34. A compound identified by the method of claim 33.

35. A therapeutic composition comprising the compound of claim 34 and a therapeutically acceptable carrier.

36. A method of determining the sequence specificity of a post translational type activity comprising:

a) contacting a sample containing said post translational type activity with a library of engineered optical probes, each said engineered optical probe comprising:

a first probe moiety and a fluorescent quencher attached to a polypeptide,

wherein said polypeptide comprises a recognition motif for a post-translational type modification activity, and a protease site located within said polypeptide; and

wherein post translational type modification of said recognition motif within said polypeptide by said post-translational type modification activity modulates the rate of a protease with specificity for said protease site to cleave said polypeptide, and

wherein said recognition motif and said protease site are located between said first probe moiety and said fluorescent quencher,

b) contacting said sample containing said post translational type activity and said engineered optical probe with a protease, and

c) determining an optical property of each of said library of said engineered optical probes.

37. A therapeutic composition identified by the method for determining whether a test chemical modulates the activity of a post translational type modification activity, comprising the steps of:

a) contacting a sample containing an enzyme with a test compound and an optical probe, said optical probe comprising:

a first probe moiety attached to a polypeptide,

wherein said polypeptide comprises a recognition motif for a post-translational modification enzyme, and

a protease site located within said polypeptide; and

wherein post translational type modification of said recognition motif within said polypeptide by said post-translational type modification activity modulates the rate of a protease with specificity for said protease site to cleave said polypeptide,

b) contacting said sample containing an enzyme with a test compound and said optical probe with a protease, and

c) determining at least one optical property of said optical probe,

d) monitoring the toxicology of said therapeutic in an *in vitro* or *in vivo* model.

38. The therapeutic composition of claim 37,

wherein said method further comprises the step of monitoring the efficacy of said therapeutic in an *in vitro* or *in vivo* model.

39. A system for spectroscopic measurements, comprising:

at least one reagent for an assay and a device, said device comprising a container and a platform,

wherein said container comprises

a) an optical probe, comprising: a first probe moiety attached to a polypeptide,

wherein said polypeptide comprises a recognition motif for a post-translational modification activity, and a protease site located within said polypeptide; and

wherein post translational type modification of said recognition motif within said polypeptide by said post-translational type modification activity modulates the rate of a protease with specificity for said protease site to cleave said polypeptide,

b) a sample,

c) a protease with specificity for said protease site.

40. The system of claim 39, wherein said container is a well in a multiwell plate.

41. A system for microfluidic spectroscopic measurements, comprising:

1) a device, said device comprising a fluid filled structure with at least one electro-osmotic or electrophoretic system to control fluid movement within said device,

2) an optical probe, comprising: a first probe moiety attached to a polypeptide,

wherein said polypeptide comprises a recognition motif for a post-translational modification activity, and a protease site located within said polypeptide; and

wherein post translational type modification of said recognition motif within said polypeptide by said post-translational type modification activity modulates the rate of a protease with specificity for said protease site to cleave said polypeptide,

3) at least one sample, and

4) a protease with specificity for said protease site.

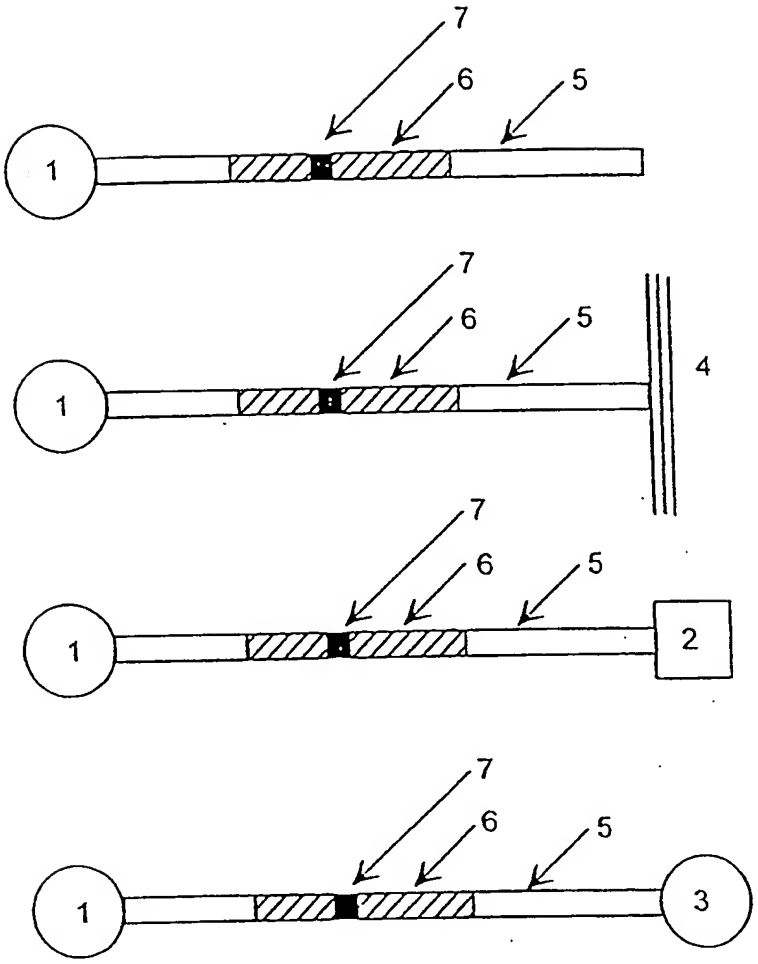

FIG. 1

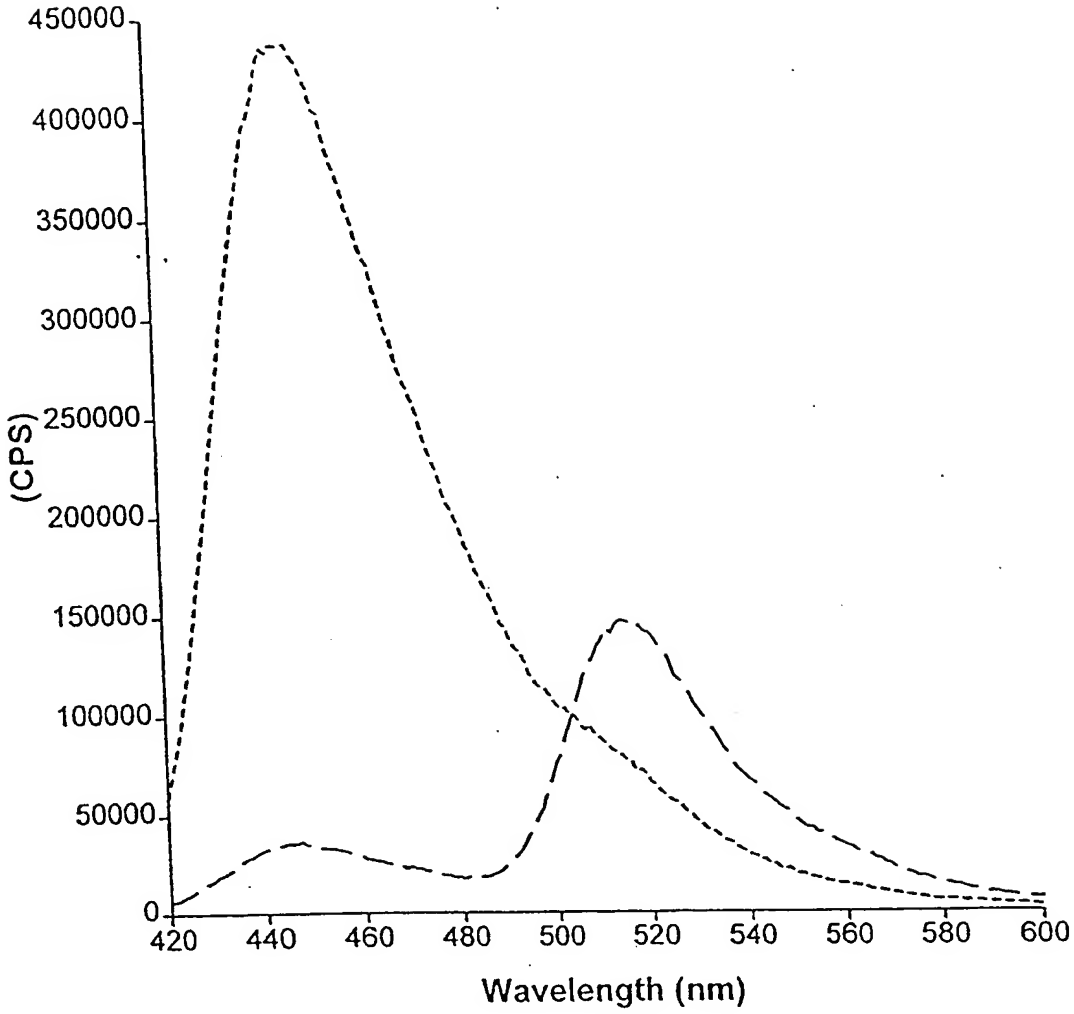

FIG. 2

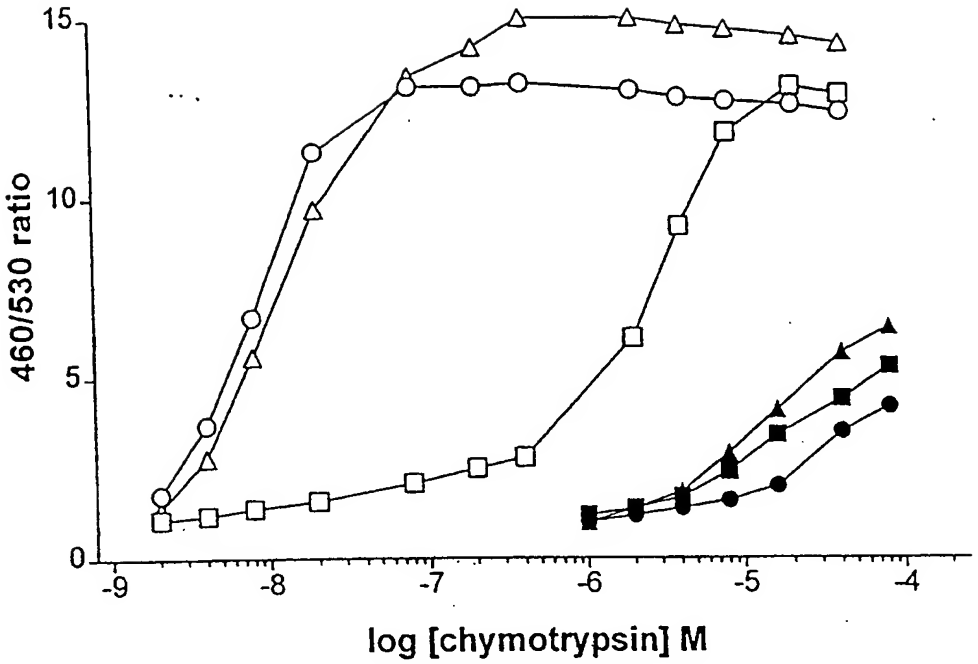

FIG. 3

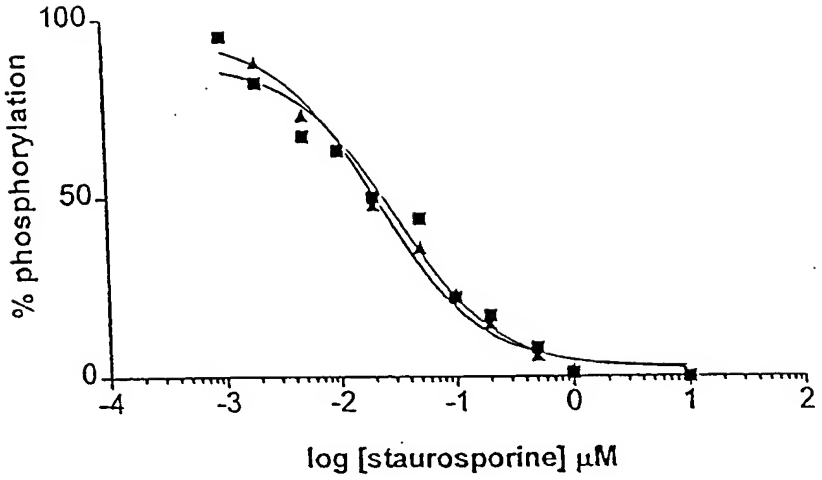

FIG. 4

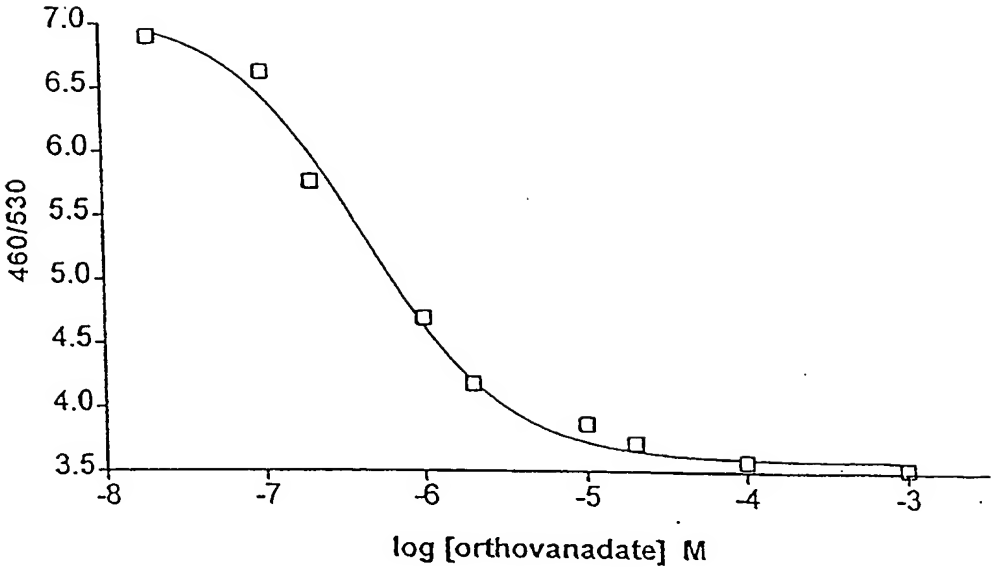

FIG. 5

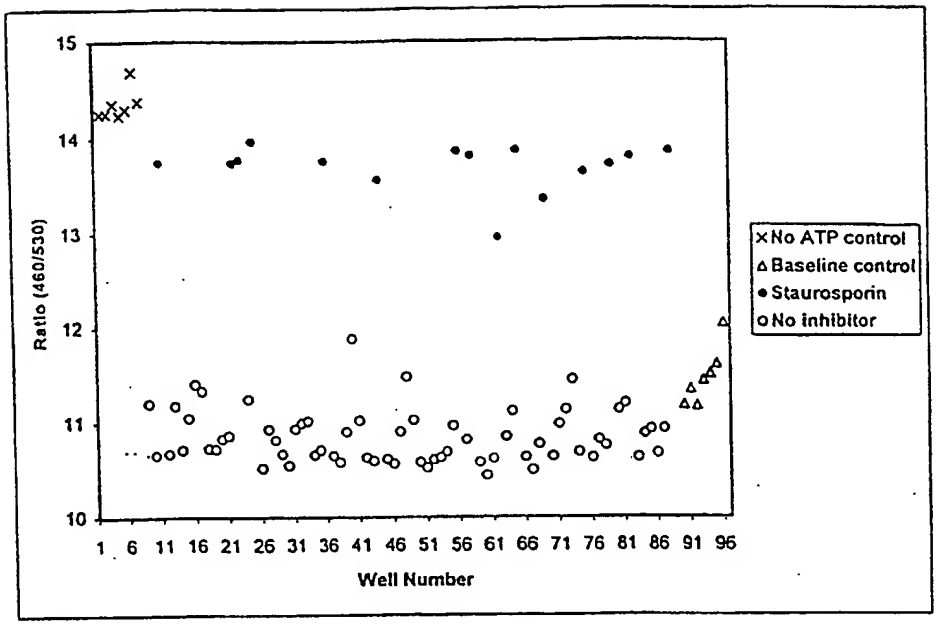

FIG. 6

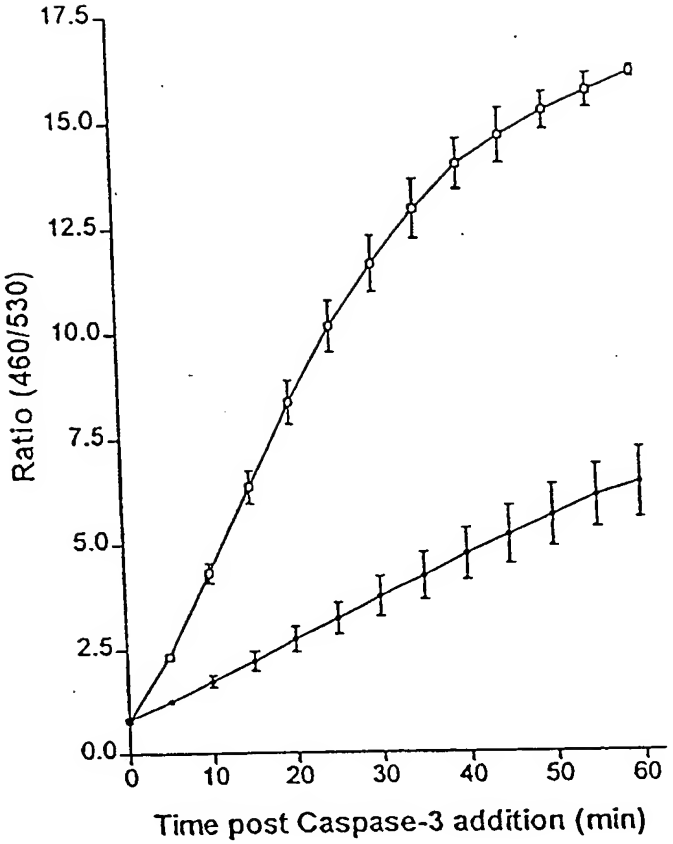

FIG. 7

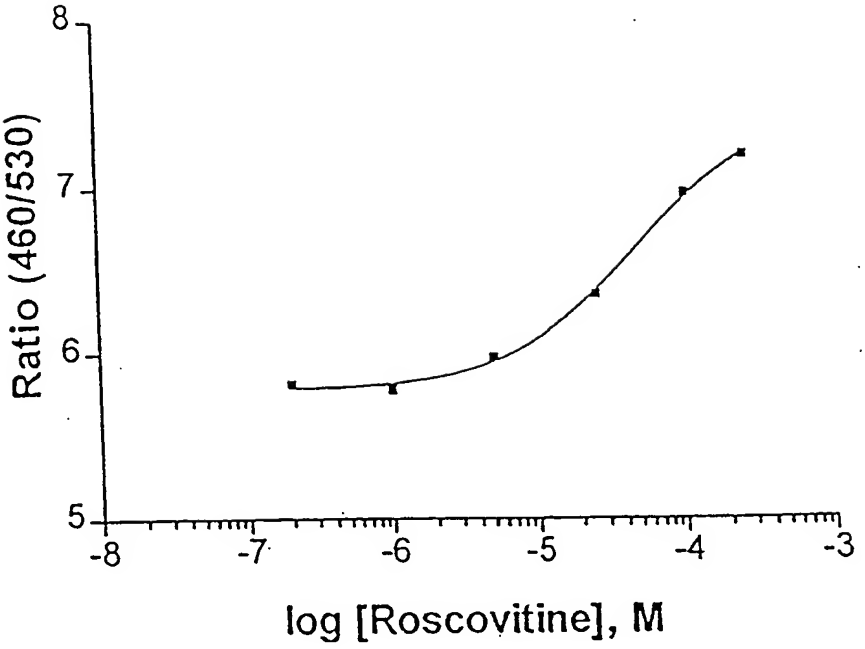

FIG. 8

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/37 C12Q1/42 C12Q1/48 C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 11251 A (ABELL CHRISTOPHER ;BALASUBRAMANIAN SHANKAR (GB); UNIV CAMBRIDGE TE) 19 March 1998 (1998-03-19) the whole document	1-33,36, 39-41
A	US 5 773 237 A (MCCORMICK FRANCIS P ET AL) 30 June 1998 (1998-06-30) abstract claims	1-33,36, 39-41

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 September 2000	09/10/2000

Name and mailing address of the ISA	Authorized officer
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Form PCT/ISA/210 (second sheet) (July 1992)

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